

protein kinase (Sgk) by recognition of a nuclear  
localization signal in the kinase central domain

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## ABSTRACT

The transcriptionally regulated serum and glucocorticoid inducible protein kinase (Sgk) is localized to the nucleus in a serum dependent manner, and a yeast two hybrid genetic screen uncovered a specific interaction between Sgk and the importin- $\alpha$  nuclear import receptor. In vitro GST pull down assays demonstrated a strong and direct association of importin- $\alpha$  with endogenous Sgk and exogenously expressed HA-tagged Sgk, while both components co-immunoprecipitate, and co-localize to the nucleus following serum stimulation. Consistent with an active mechanism of nuclear localization, the nuclear import of HA-Sgk in permeabilized cells required ATP, cytoplasm and a functional nuclear pore complex.

Mutagenesis of lysines by

alanine substitution defined a KKAILKKKEEK sequence within the central domain of Sgk between amino acids 131-141 that functions as a nuclear localization signal (NLS) required for the in vitro interaction with importin- $\alpha$ , and for nuclear import of full length Sgk in cultured cells. The serum induced nuclear import of Sgk requires the NLS-dependent recognition of Sgk by importin- $\alpha$  as well as the PI3-kinase-dependent phosphorylation of Sgk. Our results define a new role importin- $\alpha$  in the stimulus dependent control of signal transduction by nuclear localized protein kinases.

## INTRODUCTION

Protein kinases are an important class of regulatory molecules that act as communication channels to transduce signals from the cell surface to intracellular sites to control a wide range of complex biological processes through the rapid and reversible phosphorylation of specific substrates (Karin and Hunter, 1995; Hunter, 2000). Though the vast majority of protein kinases are regulated predominantly by their posttranslational phosphorylation and dephosphorylation to control their enzymatic activity (Hunter, 1995, 2000), some kinases can also be controlled by specific inhibitory and stimulatory molecules, the control of expression, or alterations in subcellular localization, or the control of expression (Simmons *et al.*, 1992; Clay *et al.*, 1993; Holtrich *et al.*, 1994; Donohue *et al.*, 1995; Hollister *et al.*, 1997; Hunter, 2000). An emerging group of protein kinases require their active translocation into the nucleus for complete exertion of their cellular effects (Mizukami *et al.*, 1997; Khokhlatchev *et al.*, 1998; Lenormand *et al.*, 1998; Hulleman *et al.*, 1999; Borgatti *et al.*, 2000). Anchoring proteins and nuclear tethers have been suggested to explain the cytoplasmic or nuclear retention of protein kinases such as Erks/MAPK and PKA (Pawson and Scott, 1997; Blanco-Aparicio *et al.*, 1999; Brunet *et al.*, 1999; Klussmann *et al.*, 1999; Edwards and Scott, 2000; Cyert, 2001). However, for most nuclear localized protein

kinases, relatively little is known about the precise cellular processes and components responsible for their nuclear import. To address the issues of multi-level cellular control of protein kinases, we have been investigating the stimulus dependent control of transcription, enzymatic activity and nuclear-cytoplasmic localization of the serum and glucocorticoid inducible serine/threonine protein kinase, Sgk.

Sgk was originally isolated by subtractive hybridization from rat mammary tumor cells as a novel protein kinase that is under acute transcriptional control by both serum and glucocorticoids (Webster *et al.*, 1993a; Webster *et al.*, 1993b). The *sgk* gene encodes a 50 kDa protein and contains a catalytic domain that is approximately 45-55% homologous to the catalytic domains of several well characterized serine/threonine protein kinases that are constitutively expressed such as Akt/PKB, protein kinase A, protein kinase C-zeta and the rat p70<sup>S6K</sup>/p85<sup>S6K</sup> kinases (Webster *et al.*, 1993b). More recently, various studies have provided substantial evidence implicating a role for Sgk in transducing signals vital for cell survival and proliferative responses (Webster *et al.*, 1993b; Buse *et al.*, 1999; Brunet *et al.*, 2001; Mikosz *et al.*, 2001; Xu *et al.*, 2001), in the control of epithelial sodium channel activity and sodium homeostasis (Alvarez de la Rosa *et al.*, 1999; Chen *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999; Shigaev *et al.*, 2000; Kamynina and Staub, 2002), and in the nephropathy associated with the diabetic disease state (Kumar *et al.*, 1999; Reeves and Andreoli, 2000). It is now recognized that Sgk gene transcription can be acutely regulated by hormonal, mitogenic and cellular stress signals in a cell type and stimulus dependent manner (Webster *et al.*, 1993a; Webster *et al.*, 1993b; Imaizumi *et al.*, 1994; Maiyar *et al.*,

1996; Alliston *et al.*, 1997; Delmolino and Castellot, 1997; Maiyar *et al.*, 1997; Waldegger *et al.*, 1997; Chen *et al.*, 1999; Iyer *et al.*, 1999; Naray-Fejes-Toth *et al.*, 1999; Waldegger *et al.*, 1999; Bell *et al.*, 2000; Brennan and Fuller, 2000; Cowling and Birnboim, 2000; Shigaev *et al.*, 2000; Mizuno and Nishida, 2001).

In response to serum growth factors (Buse *et al.*, 1999; Park *et al.*, 1999), insulin (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Perrotti *et al.*, 2001), oxidative stress (Kobayashi and Cohen, 1999; Park *et al.*, 1999), and hyperosmotic conditions (Bell *et al.*, 2000), Sgk enzymatic activity and phosphorylation is regulated as a downstream component of the phospho-inositide 3-kinase (PI 3-kinase) cascade, which results in the activation of the phosphatidyl dependent protein kinase-1, PDK1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999). Stimulation of Sgk enzymatic activity results from two key phosphorylation events at residue threonine 256 in the activation loop of Sgk and residue serine 422 that are directly targeted by PDK-1 enzyme and presumably PDK-2 respectively (Kobayashi and Cohen, 1999; Park *et al.*, 1999). By screening a peptide library, consensus Sgk substrate sites have been identified that are generally similar to the Akt/PKB enzymatic specificity and suggest some overlap in protein substrates (Kobayashi and Cohen, 1999; Park *et al.*, 1999). Recently, Sgk has been shown to phosphorylate glycogen synthase kinase-3 (GSK-3), Raf kinase, and the forkhead family member FKHL1, in vitro (Kobayashi and Cohen, 1999; Brunet *et al.*, 2001; Zhang *et al.*, 2001), which are also targets of the Akt/PKB protein kinase, although to date the identification of Sgk substrates *in vivo* have remained elusive.

Another important level of cellular control on Sgk is the regulated subcellular distribution between the nucleus and the cytoplasm that is stringently controlled in a stimulus dependent manner in mammary epithelial cells and ovarian cells (Buse *et al.*, 1999; Gonzalez-Robayna *et al.*, 1999; Alliston *et al.*, 2000; Bell *et al.*, 2000). In serum stimulated mammary epithelial tumor cells, Sgk shuttles between the nucleus and cytoplasm in synchrony with the phase of the cell cycle in that Sgk is predominantly cytoplasmic in G1 and resides in the nucleus during the S and G2/M phases of the cell cycle (Buse *et al.*, 1999). Treatment with glucocorticoids which induces a G1 cell cycle arrest (Buse *et al.*, 1999), or exposure to hyperosmotic stress (Bell *et al.*, 2000), results in a strictly cytoplasmic form of Sgk. In the ovarian system, upon treatment with follicle stimulating hormone (FSH), Sgk resides in the nucleus of proliferating granulosa cells, while in terminally differentiated luteal cells, Sgk is located in the cytoplasmic compartment (Gonzalez-Robayna *et al.*, 1999; Alliston *et al.*, 2000). Taken together, these results strengthen the view that the spatial/temporal regulation of Sgk is vital for executing complex growth and differentiation programs, and suggests the existence of specific regulatory mechanisms for localizing Sgk to distinct cellular compartments.

Conceivably, the signal dependent compartmentalization of Sgk in different subcellular locations entails interactions with specific cellular proteins that regulate the accessibility of Sgk to its protein targets. As an initial step to understand the cellular functions of Sgk, a yeast two hybrid assay was performed to uncover Sgk interacting proteins. The present study describes the identification and functional characterization of importin- $\alpha$  as a Sgk interacting protein that controls the stimulus dependent nuclear

import of Sgk. Importin- $\alpha$  is an adapter protein that directs the NLS-driven nuclear import of protein cargoes through the nuclear pore complex (Gorlich and Mattaj, 1996; Gorlich, 1998; Christophe *et al.*, 2000; Jans *et al.*, 2000; Sweitzer *et al.*, 2000). Our studies demonstrate the existence of a NLS within the central domain of the Sgk protein that mediates the interaction with importin- $\alpha$  and, along with the requirement for phosphorylation, is required for efficient nuclear targeting of this kinase. Thus, our results establish the mechanistic basis for the stimulus dependent nuclear import Sgk, and define a new cellular role for importin- $\alpha$  in the serum induced delivery of the serine/threonine protein kinase, Sgk, into the nucleus.

## MATERIALS AND METHODS

### *Yeast Two Hybrid Screen*

The yeast two hybrid screening procedure was essentially carried out as previously described (Gyuris *et al.*, 1993) using the Match Maker Lex A-yeast two hybrid kit, which was obtained from Clonetech Laboratories Inc. (Palo Alto, CA) and contains the yeast strain EGY48 and appropriate vectors. To construct the Sgk bait plasmid, the full length Sgk cDNA was PCR cloned using EcoR1/Xho1 sites, to the 3' of yeast Lex A DNA binding domain in the his+ plasmid vector pLexA to generate the bait plasmid pLexA-Sgk. This plasmid was evaluated for inappropriate transcriptional activation of the reporter genes

LEU2 and lacZ in the yeast strain EGY48/pSH18-34 as described elsewhere (Estojak et al., 1995). All yeast transformations were performed with the Yeastmaker yeast transformation kit (Clonetech) according to the manufacturer's instructions using the lithium acetate procedure. The transformants were selected on appropriate yeast dropout media also purchased from Clonetech. Adult rat brain cDNA plasmid library expressing recombinant clones (~1 kb average size inserts) as fusion proteins with the B42 transcriptional activation domain under the direction of the GAL1 promoter within the pB42AD vector, was procured from Origene Technologies Inc., MD. The yeast strain EGY48 containing the reporters LexAop-LEU and LexAop-lacZ and expressing the bait plasmid pLexA-Sgk were transformed with 1 µg of adult rat brain cDNA using the lithium acetate method. Yeast colonies that demonstrated galactose dependent activation of both reporters (blue staining in the presence of X-Gal[5-bromo-4chloro-3-indolyl-beta-D-galactopyranoside] and leucine independent growth) were selected and considered for further evaluation to screen for putative Sgk interacting proteins.

Library plasmid DNA was isolated from these blue colonies by glass bead lysis, and rescued into KC8 E.coli strain by electroporation and recovered the transformants on minimal M9 selective medium (Amp+) lacking tryptophan (Gyuris et al., 1993). Three colonies were randomly chosen from each clone and the specificity of the interaction was tested by retransforming the interactor plasmid into yeast expressing pLexA-Sgk specific bait as well as yeast strain containing two unrelated bait plasmids pLexA-bicoid and pLexA-lamin. Interactions were judged specific only if galactose dependent transcriptional activation of LEU2 and lacZ reporters were observed in strains expressing

the Sgk bait plasmid and not with the other two irrelevant bait plasmids. The cDNA encoding specific Sgk interacting proteins were sequenced (DNA sequencing Facility, UC Berkeley).

***Generation and expression of recombinant GST-full length importin- $\alpha$  (GST- $imp\alpha$ ) and GST-truncated importin- $\alpha$  (GST- $\Delta 1-422$ ) fusion proteins***

Full length mouse importin- $\alpha$  cDNA subcloned into pGEX-3X (denoted) was a kind gift from Marian Waterman (UC Irvine, CA). The truncated importin- $\alpha$  isolated from the yeast two hybrid screen encodes the carboxy -terminal terminal 107 amino acids, and was subcloned into EcoR1/Xho1 sites within the Glutathione-S-Transferase (GST) vector pGEX-4T1 (Amersham Pharmacia Biotech) to yield the GST- $\Delta 1-422$  plasmid. The GST-importin- $\alpha$  fusion proteins were isolated from the bacterial strain AB1899 cells transformed with purified GST- $imp\alpha$  and GST- $\Delta 1-422$  expression plasmids. Bacteria were initially grown at 37°C for two hours (O.D.=0.5-0.7) and subsequently induced with 0.1 mM IPTG (isopropyl1-thio-beta-Dgalactopyranoside) for 6 hours at 37°C. Cells were lysed using the French Press (three times) in lysis buffer (PBS containing 0.05% Tween 20, 2 mM EDTA, 1 mM DTT and 0.1% beta-mercapto-ethanol). The GST-importin- $\alpha$  fusion proteins were purified on glutathione sepharose beads (Pharmacia) according to manufacturer's instructions.

***Binding of GST- $imp\alpha$  and GST- $\Delta 1-422$  to in vitro translated [ $^{35}$ S]Sgk by GST pull down assays***

In vitro transcription and translation of full length wild type Sgk (Wt Sgk), kinase dead Sgk (K127M Sgk), N-terminal and C-terminal deleted Sgk ( $\bullet$ N Sgk,  $\bullet$ C Sgk), catalytic domain of Sgk (Cat 60-355 Sgk), and truncated fragments of the Sgk central catalytic domain (60-157 Sgk) and (66-122 Sgk) subcloned into pCDNA3 vectors or pCite vectors were performed using the TNT coupled rabbit reticulocyte kit (Promega Corporation) according to manufacturer's instructions. The expression plasmids encoding the Jnk protein and PKC-zeta (PKC- $\zeta$ ) were kindly provided by J.S. Gutkind (Molecular signaling unit, National Institute of Dental Research, NIH).

To test the binding between the GST-importin- $\alpha$  fusion proteins and the in vitro translated [ $^{35}$ S]Sgk, GST-imp $\alpha$  or GST- $\Delta$ 1-422 immobilized on glutathione sepharose beads was incubated with 2-5  $\mu$ l of [ $^{35}$ S]Sgk in vitro translation product in 180  $\mu$ l of binding buffer (20 mM Hepes-KOH, pH 7.9, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM DTT, 0.2% NP-40, 1.5 mM PMSF, and 3  $\mu$ l of normal goat serum/180 $\mu$ l binding buffer). The slurry was incubated overnight at 4°C on a nutator, and then the beads were washed five times in wash buffer (200 mM NaCl, 0.2% Tween 20, 10 mM Tris pH 7.5 and 0.5% non-fat dry milk). After removing the supernatant fraction in the final wash, samples were resuspended in 25  $\mu$ l of 2x SDS sample buffer, boiled and resolved by SDS-PAGE. Binding was compared to 10% of the in vitro translated products added to the binding reactions. Gels were dried at 60 °C and autoradiography carried out at -70 °C.

***Binding of GST-*imp*α and GST-Δ1-422 to endogenous Sgk or ectopically expressed Sgk in cell extracts by GST pull down assays***

The expression of endogenous Sgk was induced in subconfluent Con8.hd6 mammary epithelial tumor cells maintained on DMEM/F12 media (Biowhittaker) containing 10% calf serum and antibiotics (pen/strep, 10ug/ml, Biowhittaker) as described previously (Webster *et al.*, 1993b). Cells were serum starved for 72 hours and subsequently pulsed with either 10% calf serum for four hours. In the indicated experiments, cells that were serum starved for 60 hours were pretreated with the PI3-kinase inhibitor LY294002 (50 μM) for 12 hours, and subsequently boosted for 4 hours with 10% calf serum replenished with fresh LY294002 inhibitor (Park *et al.*, 1999; Bell *et al.*, 2000).

The construction of mammalian expression plasmids encoding wild type Sgk, kinase dead Sgk, phosphorylation deficient single mutants, T256A Sgk and S422A Sgk, double T256A/S422A mutant, and T256D/S422D Sgk in pCMV5 vectors containing a N-terminal haemagglutinin (HA) epitope tag have been previously described (Park *et al.*, 1999). The substitution of lysine residues with alanine within the putative NLS of Sgk bearing the sequence KKAILKKKEEK (amino acid residues 131-141) in the context of the full length Sgk was performed by a sequential PCR assay using standard protocols. Overlapping oligonucleotides encompassing nucleotides 452-494 of the rat Sgk cDNA sequence, incorporating the alteration of lysine with alanine were synthesized for the PCR based site specific mutagenesis experiments. The flanking upstream and downstream primers contained EcoR1 and Xho1 restriction enzyme sites for convenient cloning into pCite vectors (in vitro translations of proteins) or CMV based pCMV4 vectors containing

N-terminal HA-epitope tag for expression in mammalian cells. The incorporation of the mutations in both vectors were confirmed by DNA sequencing. Transient transfections containing 10 µg of relevant expression plasmid in combination with 10 µg of filler DNA were carried out in HEK 293 cells cultured in DMEM media supplemented with 10% fetal calf serum and antibiotics (Biowhitakker), using the calcium phosphate method (Chen and Okayama, 1988).

The cell extracts used in the GST pull down assays were prepared essentially as previously described (Buse *et al.*, 1999), with minor modifications. Cells were lysed in HEGMN buffer (25 mM Hepes, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 0.1mM EDTA, 10% glycerol, 0.1% Nonidet P-40, pH 7.9), centrifuged for 15 minutes at 14,000 rpm in a Eppendorf microcentrifuge and supernatants recovered. Recombinant GST-impα or GST-Δ1-422 immobilized on glutathione sepharose beads was incubated with the respective cell extracts overnight at 4°C on a nutator. The beads were washed and resolved by SDS-PAGE (7.8%) gels, transferred to nitrocellulose membranes and probed for Sgk protein by anti-Sgk immunoblotting as described previously (Buse *et al.*, 1999; Bell *et al.*, 2000), or for the presence of HA-tagged proteins by anti-HA immunoblotting (Park *et al.*, 1999).

### ***Co-Immunoprecipitation of Importin-α and Sgk***

The co-immunoprecipitations of endogenous serum stimulated Sgk and overexpressed importin-α were performed in Con8.hd6 mammary epithelial cells. The full length mouse importin-α cDNA was subcloned into EcoR1/Xho1 sites within the pCMV4 vectors

designed to carry an N-terminal HA-epitope tag using standard PCR cloning methods. Cultures of 50-60% confluent cells were transfected with 2 µg of full length importin- $\alpha$  expression construct (HA-Imp $\alpha$ ) and 20 µg of lipofectamine reagent (Life Technologies, BRL) according to the manufacturer's instructions. Cells were serum starved for 36 hours, then pulsed with 10% calf serum for 4 hours to stimulate Sgk expression, and subsequently harvested. Cell lysates prepared in HEGMN buffer were precleared with protein A-Sepharose containing 10 µg of rabbit IgG, and serum activated Sgk present in the lysates immunoprecipitated with anti-Sgk polyclonal antibodies and 25 µl of protein A-Sepharose, overnight at 4°C on a nutator platform. Following three washes of the immune complexes with the wash buffer described in previous section, samples were boiled, and resolved on 7.8% SDS-PAGE gels. Anti-HA western blots were performed to detect the presence of co-immunoprecipitating HA- importin- $\alpha$  using monoclonal antibodies directed against the HA-epitope (CloneMMS-101R, Babco, Richmond CA) as described above.

Reciprocal co-immunoprecipitations of exogenously expressed importin- $\alpha$  and catalytic domain of Sgk were performed in cell extracts prepared from transfected Hek 293 cells. Expression plasmids (10 µg each) encoding full length importin- $\alpha$  (HA-IMPA) along with Cat Sgk (catalytic domain of Sgk in pCDNA3 vector) were transiently transfected into HEK 293 cells (~70% confluent) as described above using the calcium phosphate method. Cells were harvested 48 hours post-transfection and the cell lysates prepared using the HEGMN buffer detailed in the preceding section. Cell lysates (~2 mg) were precleared

with mouse IgG (~10 µg) protein G-Sepharose, and importin- $\alpha$  protein from the lysates was immunoprecipitated with anti-HA monoclonal antibodies (15 µg, 12CA5, Boehringer Mannheim), and 25 µl of protein G-Sepharose, overnight at 4°C on a nutator. The immune complexes were washed three times in the wash buffer used for GST pull down assays described above and complexes solubilized by boiling in SDS-PAGE sample buffer. Proteins were separated on 10% SDS-PAGE gels and presence of bound catalytic Sgk protein determined by anti-Sgk immunoblotting as described above.

#### *Double immunofluorescence Microscopy*

Con8.hd6 mammary epithelial tumor cells were plated at low confluence (~30%) on 2-well Lab-Tek Permanox slides (Nalgene Nunc International, Naperville, Illinois) and transiently transfected with full length importin- $\alpha$  expression plasmid (HA-IMPA) using the lipofectamine procedure as detailed in the previous sections. Cells were serum starved for 36 hours and subsequently treated with 10% calf serum for 15 hours. To incubate cells with the LY294002 PI3-kinase inhibitor, serum starved cells were pretreated with 50 µM LY294002 for 8 hours and then serum boosted in the presence of LY294002 for 15 hours. At no time did cell confluence exceed 60%, a factor deemed extremely crucial for observing the serum induced nuclear translocation of Sgk. Cells were fixed, permeabilized, and processed for indirect double immunofluorescence microscopy as described previously with minor modifications (Buse *et al.*, 1999). Cells were blocked in normal goat serum diluted (1:30) in PBS, for 15 minutes and incubated with affinity purified, anti-Sgk antibodies at 1:150 dilution, in combination with 1:1000 dilution of

murine anti-HA monoclonal antibodies (clone MMS101R, Babco, Richmond, CA) for 1-2 hours at room temperature on a rocking platform. Washed slides were incubated for 1 hour at room temperature with 1:150 dilutions of both anti-rabbit fluorescein isothiocyanite-conjugated secondary antibody (Mol. Probes Inc., Eugene OR) and Texas red-conjugated goat anti-mouse secondary antibody (Mol. Probes Inc., Eugene OR). Slides were washed and then mounted with Vectashield mounting medium (Vector Laboratories Inc.) and examined using Zeiss Axiophot optics. The assay conditions for monitoring the serum dependent localization of ectopically expressed HA-tagged wild type Sgk and the mutants that include the kinase dead Sgk (HA-K127M Sgk), single phosphorylation site mutants (HA-T256A Sgk, HA-S422A Sgk), double phosphorylation mutant (HA-T256A/S422A Sgk), constitutively phosphorylated mutant (HA-T256D/S422D Sgk), and the putative Sgk NLS mutant (HA-NLS mut Sgk) were essentially as detailed above, using anti-HA antibodies to monitor the fluorescence pattern of these exogenously expressed proteins.

### *Fluorescence Microscopy*

Coding sequences of full length Sgk were fused in frame to the C-terminus of the green fluorescence protein (GFP) protein contained in the C2-GFP expression vector (Clonetech, Palo Alto, CA) that was kindly provided by J. Richards (Dept. Molecular and Cell Biology, Baylor College of Medicine). Low confluence (~30%) Con8.hd6 mammary epithelial cells grown on 2-well Lab-Tek slides were transfected with the pGFP-Sgk encoding expression plasmid or expression plasmid encoding GFP protein alone (pC2-GFP) using Lipofectamine reagent as detailed in the preceding sections. Following serum starvation

for 36 hours, appropriate treatments with serum were carried out essentially similar to the immunofluorescence experiments described above.

### ***Nuclear Import Assays***

Hek 293 fibroblasts were transiently transfected with expression plasmids encoding the wild type HA-Sgk, using calcium phosphate method as described in the previous sections. The S-100 cytosol extract containing the exogenously expressed Sgk import substrate and used to reconstitute the nuclear transport was prepared essentially as described (Adam *et al.*, 1990). Transfected HEK 293 cells were harvested by scraping, washed twice in PBS, and once in wash buffer (10 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT). Cells were resuspended in equal volume of lysis buffer (5 mM Hepes-KOH pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, and 1 µg/ml Pepstatin) and incubated on ice for 10 minutes, followed by Dounce homogenization (pestle A). The homogenate was clarified by centrifugation (100,000 x g, 1 hr, 4°C), dialyzed for several hours against transport buffer using collodion membranes (MWCO 25,000, Schleicher and Schuell Inc., Keene NH), and stored at -80°C. Cytosols enriched in serum activated endogenous Sgk from mammary epithelial cells were prepared essentially as above.

The digitonin permeabilization of Hela cells and the in vitro nuclear import assay was performed as described previously (Adam *et al.*, 1990; Weis *et al.*, 1996) with minor modifications. Hela cells (~50% confluent) grown on coverslips in DMEM media

supplemented with 10% fetal bovine serum and antibiotics (Biowhittaker) were rinsed in PBS three times, permeabilized in buffer containing 50 mM Hepes pH 7.3, 50 mM potassium acetate, 8 mM MgCl<sub>2</sub> and 50 µg/ml digitonin (Calbiochem, La Jolla, CA), in the presence of an energy regeneration system consisting of 20 mM creatine phosphate, 0.1 mM ATP, 0.1 mM GTP and 50 µg/ml creatine kinase for 5 minutes at room temperature. Permeabilized cells were washed three times with transport buffer (20 mM Hepes/KOH pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 2 mM DTT). The nuclear import reaction was initiated in 10 µl volume with the addition of the ATP regeneration system (20 mM creatine phosphate, 0.5 mM ATP, 0.5 mM GTP and 50 µg/ml creatine kinase), and the HEK 293 cytosols (10 mg/ml) containing the import substrates HA-Sgk, or endogenous serum activated Sgk from mammary epithelial tumor cells, and soluble import factors. The import reaction was allowed to proceed at room temperature for 30 minutes in a humidified chamber. In the indicated experiments, the ATP regeneration system was replaced with an ATP depletion mixture containing 50 U/ml of apyrase (Sigma) to block energy dependent nuclear transport pathways or wheat germ agglutinin (Calbiochem) added to a final concentration of 0.5 mg/ml to inhibit translocation via the nuclear pores.

Following the import reactions, transport of the import substrates HA-Sgk or endogenous serum activated Sgk was monitored by indirect immunofluorescence microscopy as described above. The immunofluorescence staining was performed using anti-Sgk and anti-HA antibodies, and staining was examined as described in the previous sections. Specificity of the antibody interactions were ascertained in control slides by

treatment with primary or secondary antibody alone and no significant fluorescence was displayed in any case.

## RESULTS

### *Identification of importin- $\alpha$ -1 as a Sgk interacting protein*

A LexA-based yeast two hybrid screen (Gyuris *et al.*, 1993) was undertaken to identify proteins that interact with Sgk. Approximately 10 million yeast transformants were screened based on their galactose dependent transcription of leucine and lacZ reporter genes. Among several putative interacting clones, three isolates contained cDNA inserts of similar size (~0.5 kb). Colonies of the EGY48-derived yeast strain transformed with each of these library derived plasmids (clone 1, clone 2, and clone 3) exhibited growth on galactose based media devoid of leucine, tryptophan, uracil, histidine in the presence of the pLex-Sgk bait plasmid (Figure 1A). In contrast, none of the yeast colonies expressing the library derived plasmids were able to grow upon transformation with either pLex-Lamin or pLex-Bicoid, transcriptionally inert non specific baits (Figure 1A, middle and lower panels).

BLAST analysis revealed that all three library clones are the rat homologues of the mouse importin- $\alpha$ -1 gene (Genbank TM accession number U12270, U34229, D55720). As shown in Figure 1B, mouse importin- $\alpha$ -1 is a 58 kDa protein that contains a hydrophobic

amino-terminus, a conserved central hydrophilic region punctuated with 8-10 degenerate arm repeats and a short hydrophilic carboxy-terminus that is enriched in highly acidic amino acid residues. This acidic region at the carboxy-terminus has been shown in some cases to interact with the NLS in cargo proteins that are destined for the nucleus (Prieve *et al.*, 1996; Prieve *et al.*, 1998). Sequence analysis demonstrated that the isolated library clones, which interact with Sgk, show near 98% sequence homology to the mouse gene and encode the carboxy-terminal 107 amino acids (residues 423-529) of rat importin- $\alpha$ -1. This cloned region encompasses approximately half of the eighth arm repeat and the entire ninth repeat extending up to the predicted carboxy-terminal region (Figure 1B, truncated importin- $\alpha$ ). For simplicity, we allude to the library derived clones or the full length cDNA as importin- $\alpha$ .

***Specificity of the interaction of Sgk with GST-linked full length and truncated importin- $\alpha$***

In vitro GST pull down assays were utilized to confirm biochemically the fidelity of the interaction between Sgk and importin- $\alpha$  observed in yeast. Wild type [ $^{35}$ S]Sgk was in vitro translated and analyzed for its ability to bind either the full length importin- $\alpha$  (GST-imp $\alpha$ ) or the library derived truncated importin- $\alpha$  (GST- $\Delta$ 1-422) expressed as GST fusion proteins. The GST fusion proteins, as well as the GST protein, immobilized on glutathione sepharose beads were incubated with [ $^{35}$ S]Sgk, and binding to the beads was determined by fractionation in SDS-PAGE and visualization by autoradiography. The programmed lysate containing the in vitro translated [ $^{35}$ S]Sgk represents the input used in the importin- $\alpha$  binding assays (Figure 2A). As also shown in Figure 2A, [ $^{35}$ S]Sgk associated with both

full length importin- $\alpha$  (GST-imp $\alpha$ ) and truncated importin- $\alpha$  (GST- $\Delta$ 1-422), and not with GST alone. Furthermore, unlabeled in vitro translated Sgk effectively competed off [ $^{35}$ S]Sgk from GST-imp $\alpha$  in the GST pull down assays (data not shown). Taken together, our results show that Sgk directly binds importin- $\alpha$  and that the carboxy-terminal portion of importin- $\alpha$  is sufficient to mediate this interaction.

GST pull down assays were utilized to examine the interaction of importin- $\alpha$  with other protein kinases that are either related or unrelated to Sgk, and which display signal dependent nuclear translocation. Protein Kinase C- $\zeta$  (PKC- $\zeta$ ) is 45% homologous to the catalytic domain of Sgk and activated by PI 3-kinase signaling, whereas Jun N-terminal Kinase (Jnk) is unrelated to Sgk. The mechanism of nuclear import for each of these protein kinases is largely unknown (Mizukami *et al.*, 1997; Borgatti *et al.*, 2000). As shown in Figure 2B, in vitro translated [ $^{35}$ S]PKC- $\zeta$  and [ $^{35}$ S]Jnk both fail to interact with full length importin- $\alpha$  under the same conditions in which [ $^{35}$ S]Sgk efficiently binds to importin- $\alpha$ , further demonstrating the specificity of the Sgk-importin- $\alpha$  interaction.

To biochemically validate that endogenous Sgk interacts with importin- $\alpha$ , GST pull down assays were carried out using full length recombinant importin- $\alpha$  (GST-imp $\alpha$ ), or the truncated importin- $\alpha$  (GST- $\Delta$ 1-422) in extracts from cells induced to by serum to produce Sgk. As observed previously (Webster *et al.*, 1993a; Webster *et al.*, 1993b; Buse *et al.*, 1999), short (4 hr) or longer (15 hr) serum treatment of 72 hour serum-starved cells stimulated high levels of Sgk protein expression (Figure 2C, -S vs +S input lanes). The

endogenous serum induced Sgk associated with both the full length and truncated importin- $\alpha$  with approximately the same efficiency as determined by anti-Sgk immunoblotting (Figure 2C). No binding was observed using beads containing GST alone.

### *Co-immunoprecipitation of Sgk and importin- $\alpha$ in mammalian cells*

Co-immunoprecipitation experiments were carried out to determine whether importin- $\alpha$  can bind to Sgk in the context of the cellular environment. To test this, HA tagged full-length importin- $\alpha$  was ectopically expressed in Con8.hd6 mammary tumor cells, and serum starved cells were then stimulated with 10% serum to induce expression of endogenous Sgk protein. The level of exogenous HA-importin- $\alpha$  produced in the cells is shown in the "Input" lane (Figure 3A, right panel). Extracts prepared from the HA-imp $\alpha$  transfected cells were immunoprecipitated with either anti-Sgk antibodies or with control non-immune rabbit antibodies. The anti-Sgk antibodies quantitatively recovered the endogenous Sgk as shown by the difference in Sgk protein in extracts prior to and following the immunoprecipitation (Figure 3A, left panel, Pre vs Post Sgk-IP). The proteins recovered in the immune complexes were subjected to SDS-PAGE analysis and the presence of importin- $\alpha$  monitored by anti-HA immunoblotting. As shown in Figure 3A, (right panels), exogenous HA-importin- $\alpha$  interacts with endogenous Sgk in the transfected cells as judged by the presence of the 58 kDa HA-importin- $\alpha$  protein only in the anti-Sgk immunoprecipitates (Figure 3A, + IP lane), but not in control immunoprecipitates (Figure 3A, - IP lane).

A reciprocal co-immunoprecipitation between Sgk and importin- $\alpha$  was carried out in Hek 293 cells co-transfected with expression vectors encoding the HA-tagged full length importin- $\alpha$  (HA-imp $\alpha$ ) and the central catalytic domain of Sgk (Cat Sgk). As detailed in a later section, the importin- $\alpha$  binding site is contained within this central domain of Sgk. Cell extracts were immunoprecipitated with anti-HA antibodies (+ IP) or with non-immune antibodies (- IP), the immunocomplexes resolved by SDS-PAGE and then immunoblotted with anti-Sgk antibodies. As shown in Figure 3B (right panel +IP vs -IP), Cat Sgk is recovered in the immune complexes containing HA-imp $\alpha$  only when immunoprecipitated with specific anti-HA antibodies, whereas, immunoprecipitation of the same cell extracts with non-immune antibodies failed to recover any Sgk protein. The expression of both Cat Sgk and HA-imp $\alpha$  proteins in transfected cells was confirmed by immunoblotting with either anti-HA or anti-Sgk antibodies (Figure 3B right panel, Input Cat Sgk vs vector control; Figure 3B left panel, Input HA-imp $\alpha$  vs vector control).

#### *Signal dependent co-localization of endogenous Sgk with exogenous importin- $\alpha$*

We have previously established that in serum stimulated cells, Sgk shuttles between the nucleus and the cytoplasm in synchrony with the cell cycle in rat Con8.hd6 mammary tumor cells (Buse *et al.*, 1999). Based on the Sgk- importin- $\alpha$  interactions demonstrated above, and on the known role of importin- $\alpha$  in nuclear import (Gorlich and Mattaj, 1996; Ferrigno *et al.*, 1998; Gorlich, 1998; Truant *et al.*, 1998; Gamblin and Smerdon, 1999; Liang and Clarke, 1999; Hood and Silver, 2000; Sweitzer *et al.*, 2000), we tested whether the

localization of importin- $\alpha$  may be linked to the signal dependent compartmentalization of Sgk.

The full length importin- $\alpha$  expression vector (HA-imp $\alpha$ ) was transiently transfected into Con8.hd6 cells, and following 48 hours of serum starvation, cells were stimulated either with 10% calf serum (+ Serum) or remained in the serum free medium (- Serum). The co-localization of importin- $\alpha$  and endogenous Sgk was monitored using double immunofluorescence microscopy; endogenous Sgk was visualized with FITC fluorescence, and the distribution of importin- $\alpha$  was denoted by Texas red staining. In serum starved cells, virtually no endogenous Sgk is produced, whereas, the ectopically expressed HA-imp $\alpha$  is localized primarily in the cytoplasmic compartment (Figure 4A, left panels). Treatment of these cells with serum caused both endogenous Sgk and recombinant importin- $\alpha$  to reside predominantly in the nucleus (Figure 4A, middle panels). Immunoblotting of the transfected cells with anti-Sgk or anti-HA antibodies confirmed that the exogenous importin- $\alpha$  is expressed under the tested conditions, whereas, as expected endogenous Sgk is produced only in the serum treated cells, but not in serum starved cells (Figure 4A, left). The signal dependent co-localization of Sgk and importin- $\alpha$  suggests that appropriate compartmentalization of Sgk may be functionally linked to the targeting activity of the nuclear import receptor importin- $\alpha$  which mediates the active transport of a variety of cellular cargoes.

**Serum dependent translocation of a 77 kDa GFP-Sgk fusion protein into the nucleus** To initially test whether Sgk is actively transported into the nucleus, an expression vector was engineered to encode the 27 kDa green fluorescent protein (GFP) fused to the N-terminus of Sgk, forming the 77 kDa GFP-Sgk. The size of this fusion protein is too large to be passively transported into the nucleus, which excludes molecules of approximately 50 kDa or larger, and therefore can negotiate the nuclear pore complex only by the active transport pathway (Kohler *et al.*, 1999a; Bayliss *et al.*, 2000). The Con8.hd6 mammary epithelial cells, transiently transfected with the GFP-Sgk expression vector, were serum starved, then boosted with 10% serum, and the subcellular distribution of GFP-Sgk was monitored by direct fluorescence microscopy. As also depicted in Figure 4B (left panels), ectopically expressed GFP-Sgk displayed strong serum inducible nuclear staining and remained cytoplasmic in the serum starved cells. Transiently transfected GFP alone was distributed throughout the cell in serum starved as well as serum stimulated cells (data not shown). As shown in Figure 4B (right panel), GFP-Sgk is expressed at comparable levels in the transfected cells in each condition, whereas, endogenous Sgk, as defined by immunoblotting with anti-Sgk antibodies, is produced only in the cell cultures incubated with serum.

***In vitro nuclear import of Sgk requires ATP, cytoplasm, and can be inhibited by a disruptor of nuclear pore complex function***

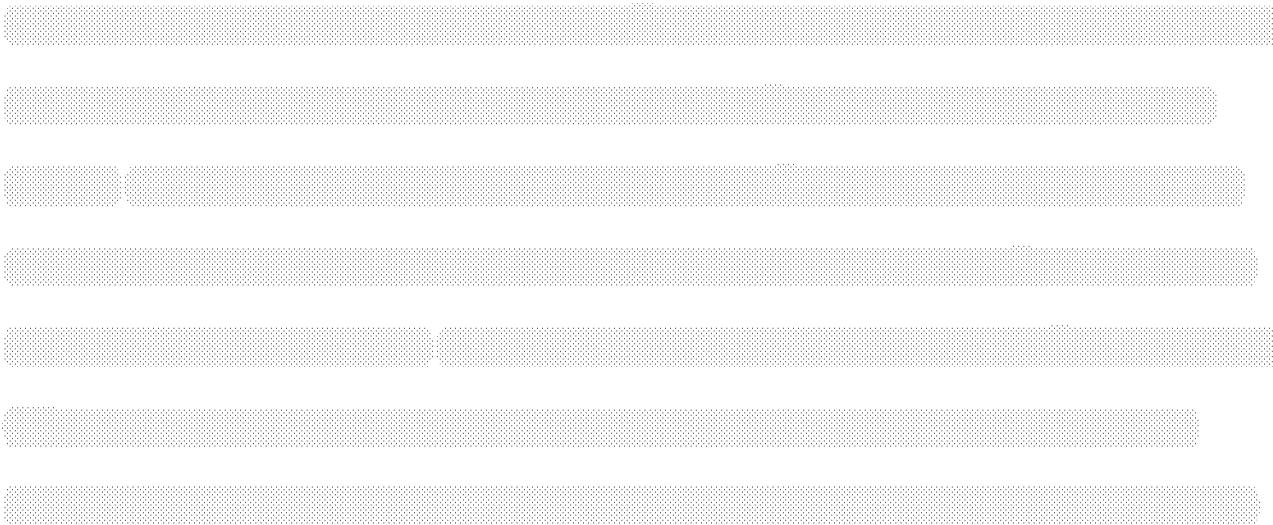
An in vitro assay was utilized to directly test whether the nuclear import of Sgk is an active process. Digitonin permeabilized Hela cell nuclei served as the source of intact nuclei for the in vitro import assays (Adam *et al.*, 1990), and the source of Sgk protein was

cytosol derived from HA-Sgk transfected Hek 293 cells. Western blots confirmed the expression of HA-Sgk in the cytoplasmic extracts used for the nuclear import assays (Figure 5B). The soluble factors such as importin- $\alpha$ , importin- $\beta$  and other components necessary for NLS-driven nuclear transport is provided by the cytoplasmic extracts. To determine whether the nuclear import of Sgk is an ATP dependent process, cytoplasmic extracts were pretreated with or without apyrase for 15 minutes in order to inhibit ATP production. An ATP regenerating system (creatinine kinase, creatine, and ATP) was added to the extracts not treated with apyrase to maintain the level of ATP. Nuclei were incubated with cytoplasmic extracts, and nuclear import of HA-Sgk was assayed by indirect immunofluorescence using anti-HA antibodies. As shown in Figure 5A (top panels), HA-Sgk was efficiently localized to the nucleus only in the presence of an ATP energy source but was impaired under energy deprived conditions. A synthetic fluorescence-tagged SV40 T-Antigen NLS peptide, conjugated to bovine serum albumin (FITC-NLS-BSA) was used as a positive control for monitoring the ATP dependent active NLS-mediated nuclear import (data not shown) (Weis *et al.*, 1996).

The in vitro assay was also utilized to examine the requirements of cytosol and an intact nuclear pore complex (NPC) for the nuclear import of HA-Sgk. As also shown in Figure 5A (middle panels), in the absence of cytosol (but containing import buffer), HA-Sgk failed to be transported into the isolated nuclei. Wheat germ agglutinin binds to and ablates the function of the nuclear pore by preventing the interactions with the nuclear cargo (Adam *et al.*, 1990). Therefore, isolated nuclei were pre-incubated with or without wheat germ agglutinin, and the nuclear import assay was carried out with cell extracts

containing the HA-Sgk. Treatment with wheat germ agglutinin completely blocked the nuclear import of HA-Sgk, compared to a parallel reaction carried out in the absence of this lectin in which HA-Sgk was efficiently localized to the isolated nuclei (Figure 5A lower panels). These data suggest that the nuclear import of Sgk proceeds by an active transport pathway, via the nuclear pore complex, requiring soluble components of the cytosol.

*The carboxy-terminal fragment of importin- $\alpha$  isolated by the yeast two hybrid screen inhibits the nuclear import of Sgk*



were incubated with either GST alone (4  $\mu$ g) or with GST- $\Delta$ 1-422 (4  $\mu$ g) along with the nuclear import cocktail and the isolated nuclei. The nuclear import of Sgk was monitored either by anti-HA or anti-Sgk immunofluorescence. As shown in Figure 6, the nuclear import of both exogenous and endogenous Sgk was significantly impaired in the presence of GST-truncated importin- $\alpha$  relative to GST alone. These results demonstrate that the active nuclear transport of Sgk is mediated by importin- $\alpha$ .

***The importin- $\alpha$  binding domain spans amino acid residues 122-157 within the central catalytic domain of Sgk***

Mutagenesis analysis of the Sgk protein was carried out to initially delimit the region within Sgk responsible for binding importin- $\alpha$ . As shown in Figure 7A, wild type Sgk is a 431 amino acid protein that contains three general domains, the 60 amino acid N-terminal region unique to Sgk, the central catalytic domain bearing a 45-55% homology to other closely related kinases, and a 76 amino acid C-terminal region that is also unique to Sgk. A kinase dead form of full length Sgk (K127M) was generated by substituting lysine 127 with methionine, rendering Sgk enzymatically inactive (Park *et al.*, 1999). Three deletions were also constructed that encoded truncated forms of Sgk that either lack the N-terminal 60 amino acids ( $\bullet$ N 61-431), the C-terminal 76 amino acids ( $\bullet$ C 1-355), or both the C-terminal and N-terminal regions of Sgk that results in a Sgk fragment spanning only the central catalytic domain (Cat 60-355). In vitro translated [ $^{35}$ S]Sgk proteins representing the input used for the importin- $\alpha$  binding assays are shown in Figure 7B, left panel. Each of the radiolabeled Sgk proteins were incubated with either GST-imp $\alpha$  or GST alone immobilized on glutathione sepharose beads, and assayed for binding by the GST pull down assay. As shown in Figure 7B (right panel), full length importin- $\alpha$  is capable of binding to the wild type, the K127M kinase dead, N-terminal truncated Sgk, C-terminal truncated Sgk or the Sgk central catalytic domain with approximately equal efficiency.

Two additional truncations within the central catalytic domain generated fragments of Sgk spanning amino acid residues 60-157 (60-157 Sgk) and amino acid residues 60-122 (60-122 Sgk), and were used to narrow down the general boundaries of the importin- $\alpha$  binding region within Sgk (see diagrams in Figure 7A). Comparison of binding of either GST-imp $\alpha$  or GST- $\Delta$ 1-422 with these two Sgk fragments revealed that the 60-157 amino acid Sgk fragment efficiently interacts with both GST-imp $\alpha$  and GST- $\Delta$ 1-422. In contrast, the 60-122 Sgk fragment appears to interact only very weakly with the full length importin- $\alpha$  and not at all with the truncated form of importin- $\alpha$  (Figure 7C, lower panel). Thus, a stretch of 35 amino acids encompassing amino acid residues 122-157 within the central catalytic domain of Sgk is very important for binding to this nuclear import component.

### *Identification of the Sgk Nuclear Localization Signal*

As shown in Figure 8A, close inspection of the 35 amino acid importin- $\alpha$  binding domain of Sgk reveals the existence of a lysine-rich region KKAILKKKEEK between amino acids 131-141 that resembles a consensus bipartite NLS (Robbins *et al.*, 1991). In order to determine whether this sequence is a functional NLS, the lysines were altered to alanines within the context of the full length Sgk to yield a NLS mutant Sgk (Figure 8A). The ability of the NLS mutant Sgk to interact with full length importin- $\alpha$  was assessed by GST pull down assays using the in vitro translated [<sup>35</sup>S]Sgk proteins as described in the preceding sections. Comparable amounts of wild type and mutant in vitro translated [<sup>35</sup>S]Sgk proteins were incubated with GST-imp $\alpha$  or GST alone and assayed for their ability

to selectively interact with importin- $\alpha$ . Efficient binding between GST-imp $\alpha$  and wild type [ $^{35}$ S]Sgk was readily apparent, whereas, the interaction between GST-imp $\alpha$  and the NLS mutant [ $^{35}$ S]Sgk was greatly attenuated and barely above the background levels of GST protein alone (Figure 8B, left panel).

In a complementary set of experiments, the ability of wild type Sgk (HA-Wt Sgk) or the NLS mutant Sgk protein (HA-NLS Mut Sgk) expressed in Hek 293 cells to interact with the full length importin- $\alpha$  was also examined using in vitro GST pull down assays. As shown in Figure 8B (right panel), the input lanes show the expression of the wild type or NLS mutant Sgk relative to vector control transfected cells. The higher molecular forms of each input protein likely represent the phosphorylated forms the wild type and NLS mutant Sgk. Anti-HA immunoblotting was used to determine the efficiency of HA-tagged Sgk interaction with the GST- importin- $\alpha$  (GST-imp $\alpha$ ) or GST protein. Consistent with the binding data observed with the in vitro translated Sgk, cell extracts containing wild type Sgk associated efficiently with the full length importin- $\alpha$ , whereas, only very weak binding is observed with the NLS mutant form of Sgk compared to the GST controls (Figure 8B, right panel).

At the cellular level, the importance of the lysine residues within the putative Sgk NLS for the serum induced nuclear translocation of Sgk was tested by transfecting mammary epithelial tumor cells with either the wild type HA-Sgk (HA-Wt Sgk) or the NLS mutant form of Sgk (HA-NLS Mut Sgk). Following serum starvation, cells were

either pulsed with 10% serum or remained serum free media and the subcellular localization of these proteins monitored by indirect immunofluorescence. In response to serum stimulation, exogenously expressed wild type HA-Sgk displayed a nuclear fluorescence (Figure 8C, + Serum) analogous to the distribution pattern observed with endogenous Sgk (see Figure 4). In contrast, the fluorescence pattern in serum stimulated cells transfected with the NLS mutant protein was confined to the cytoplasm. In serum deprived conditions, staining for both these proteins was primarily cytoplasmic (Figure 8C, - Serum). Thus, the lysine-rich KKAILKKKEEK region between amino acids 131-141 mediates the binding of Sgk to importin- $\alpha$ , and is essential for the serum induced nuclear translocation of Sgk.

***Serum-induced nuclear translocation of Sgk requires PI 3-kinase dependent phosphorylation of Sgk and is independent of Sgk kinase activity***

The phosphorylation states of protein kinases can in some instances regulate nuclear import, such as PCK-zeta (Neri *et al.*, 1999) and Erk/MAPK family members (Khokhlatchev *et al.*, 1998; Lenormand *et al.*, 1998; Hulleman *et al.*, 1999). Expression vectors encoding mutant forms of HA-tagged Sgk were utilized to test whether Sgk phosphorylation and/or kinase activity are required for importin- $\alpha$  binding in vitro and for the serum stimulated nuclear localization of Sgk in intact cells. One Sgk mutant is designed to be refractory to phosphorylation by the PI 3-kinase dependent pathway by substitution of both the threonine 256 in the activation loop and serine 422 for alanine forming HA-T256A/S422A Sgk. This mutation encodes a hypophosphorylated form of the Sgk protein that is enzymatically inactive (Park *et al.*, 1999). Another Sgk mutant

ablated the ATP binding by substitution of methionine for lysine at residue 127 (HA-K127M Sgk) thereby encoding a kinase dead form of Sgk that can be phosphorylated in a PI 3-kinase dependent manner.

The HA-T256A/S422A Sgk or HA-K127M Sgk mutants, as well as wild type Sgk, were ectopically expressed in HEK 293 cells, and the levels of expression were monitored by HA-immunoblotting (figure 9A, input vs vector control). Cell lysates were recovered for GST pull down assays using GST-importin- $\alpha$  (GST-imp $\alpha$ ) or GST alone. As shown in Figure 9A, both HA-K127M and HA-T256A/S422A Sgk bind efficiently with importin- $\alpha$ , comparable to wild type Sgk binding activity. Also, the HA-T256D/S422D Sgk binds to importin- $\alpha$  with approximately affinity as either the wild type or HA-T256A/S422A Sgk (data not shown). In a parallel set of experiments, mammary epithelial cells were transfected with either HA-wild type, HA-K127M, HA-T256A/S422A Sgk, or mutants encoding single phosphorylation sites ablated to alanine, HA-T256A Sgk or HA-S422A Sgk, which have also been shown to be enzymatically inactive (Park *et al.*, 1999). These cells were serum starved, then boosted with serum, and the subcellular localization of the wild type and mutant Sgk proteins was determined by indirect immunofluorescence as detailed in previous sections (Figure 9B). As shown in Figure 9B (top panels), HA-K127M Sgk is localized to the nuclear compartment, analogous to wild type Sgk in response to serum treatment. In contrast, although the T256A/S422A Sgk efficiently interacts with importin- $\alpha$  (Figure 9A), the fluorescence distribution pattern was confined predominantly to the cytoplasm in serum stimulated cells (Figure 9B bottom panels). Similarly the single

phosphorylation mutants of Sgk, HA-T256A and HA-S422A also show a cytoplasmic staining pattern with the addition of serum (Figure 9B middle panels). Under serum starved conditions, staining for the mutant and wild type Sgk proteins was detected exclusively in the cytoplasm. Interestingly, these results implicate a role for Sgk phosphorylation, but not its kinase activity, in the serum dependent nuclear uptake of Sgk, whereas these structural attributes within Sgk are dispensable for the in vitro interaction with importin- $\alpha$ .

As a complementary approach, the effects of the phosphorylation states of endogenous Sgk on binding to importin- $\alpha$  and serum induced nuclear localization was examined in Con8.hd6 mammary tumor cells. The PI 3-kinase dependent phosphorylation of Sgk can be ablated by treatment with the LY294002 PI 3-kinase inhibitor, whereas hyperphosphorylated Sgk is produced following addition of serum (Park *et al.*, 1999). Serum starved cells were treated with serum in the presence or absence of LY294002 (50  $\mu$ M), while control cells remained serum free. GST pull down assays using either GST-importin- $\alpha$  or GST alone were carried out in cell extracts isolated from each set of cells, and binding was determined by immunoblotting with anti-Sgk antibodies (Figure 10A). Both the hyperphosphorylated endogenous Sgk produced in serum stimulated cells and the hypophosphorylated form produced in the presence of LY294002 interacted with GST-importin- $\alpha$  but not with GST alone.

To examine the subcellular distribution of endogenous Sgk, mammary epithelial cells were serum starved and then treated with serum in the presence or absence of LY294002, and distribution of endogenous Sgk under these conditions examined by indirect immunofluorescence. Consistent with data obtained from the phosphorylation site mutants of Sgk, the serum dependent nuclear localization of endogenous Sgk is prevented in LY294002 treated cells with Sgk predominantly dispersed in the cytoplasm (Figure 10B, top right panel). In the absence of the inhibitor, hyperphosphorylated endogenous Sgk exhibited nuclear staining in serum treated cells. As expected, no endogenous Sgk can be detected in serum starved cells (Figure 10B, top panels). To further demonstrate the importance phosphorylation plays in nuclear localization, another mutant of Sgk, which has the PI 3-kinase dependent phosphorylation sites mutated to aspartic acid (HA-T256D/S422D Sgk), was used. The charge on the aspartic acids mimic phosphorylation; however, this construct has been shown to not be constitutively active (Kobayashi and Cohen, 1999; Park *et al.*, 1999). Con8.hd6 mammary epithelial cells were transfected with this construct, serum starved, then serum added in the presence of absence of LY294002. In the serum starved cells, Sgk staining appears mostly in the cytoplasm (Figure 10B, bottom left panel). After the addition of serum with or without LY294002, the distribution pattern of Sgk is seen in the nuclear compartment (Figure 10B bottom right panels), demonstrating that phosphorylation is a key structural requirement for nuclear import. Consistent with this concept, the T256A/S422A Sgk (described in Fig. 9) remains cytoplasmic in LY294002 treated and untreated cells (data not shown). Taken together, these results demonstrate that, although importin- $\alpha$  can bind both hyperphosphorylated

and hypophosphorylated forms of Sgk, the phosphorylation state of Sgk provides an additional signal for the serum dependent nuclear import of Sgk.

## DISCUSSION

A striking feature of Sgk compared to most other protein kinases is the multi-level control of its transcription, subcellular localization and enzymatic activity in stimulus dependent and tissue specific manner (Webster *et al.*, 1993a; Webster *et al.*, 1993b; Maiyar *et al.*, 1997; Buse *et al.*, 1999; Kobayashi and Cohen, 1999; Park *et al.*, 1999; Bell *et al.*, 2000; Sun *et al.*, 2000). The nuclear and cytoplasmic forms of Sgk are phosphorylated and enzymatically active (Buse *et al.*, 1999; Park *et al.*, 1999; Bell *et al.*, 2000), although relatively little was known about the mechanism of nuclear transport for Sgk. Using a yeast-two hybrid screen, we uncovered the nuclear import receptor importin- $\alpha$  as a binding partner of Sgk. Our functional studies demonstrate for the first time the involvement of importin- $\alpha$  in the stimulus specific import of Sgk into the nucleus. Moreover, a lysine-rich functional nuclear localization signal (NLS) was identified within Sgk, which along with Sgk phosphorylation, regulates the nuclear import of Sgk in a cellular context and likely controls the access of this kinase to its nuclear targets.

The GST pull down results using both full length importin- $\alpha$  and the truncated library clone demonstrate the direct and specific physical interaction between Sgk and importin- $\alpha$ . Notably, no associations between importin- $\alpha$  and Jnk and or PKC  $\zeta$  were discernible, despite the fact that these protein kinases exhibit regulated nuclear import

(Mizukami *et al.*, 1997; Neri *et al.*, 1999), emphasizing the existence of distinct nuclear import mechanisms. Most likely other importin- $\alpha$  isoforms mediate their nuclear translocation as different isoforms have been shown to display unique as well as overlapping and redundant target specificities (Kohler *et al.*, 1999b). Alternatively, the transport of these kinases may be mediated by a different set of transport receptors such as importin- $\beta$ , transportins, or importin-7 which interact with diverse array of cellular cargoes with distinct specificities (Jans *et al.*, 2000; Lorenzen *et al.*, 2001).

The selective and bidirectional nuclear-cytoplasmic trafficking of cellular proteins via the nuclear pore complex (NPC) is mediated by a family of importin/exportin shuttling transport factors that establish a controlled barrier between the cytoplasmic and nuclear compartments (Gorlich and Mattaj, 1996; Ferrigno *et al.*, 1998; Gorlich, 1998; Truant *et al.*, 1998; Gamblin and Smerdon, 1999; Christophe *et al.*, 2000; Hood and Silver, 2000; Jans *et al.*, 2000). The direction of transport depends on whether the Ran small GTPase is in its GDP or GTP bound form. This regulatory molecule controls the interactions of the nuclear pore complex with cargo, certain adapters and transport receptors (Sweitzer *et al.*, 2000). NLS-driven nuclear import requires the binding of a basic lysine-rich sequence (NLS) within a protein cargo to a importin-adapter component of the importin- $\alpha$ /importin- $\beta$  heterodimer. This NLS-protein complex docks to NPC via importin- $\beta$ , and subsequent energy dependent translocation through the pore (Gorlich and Mattaj, 1996; Gorlich, 1998; Christophe *et al.*, 2000; Jans *et al.*, 2000; Sweitzer *et al.*, 2000). A subset of protein kinases, including mammalian erk2/MAPK (Adachi *et al.*, 1999; Reiser *et al.*, 1999;

Rubinfeld *et al.*, 1999) and the Drosophila Erk proteins (Lorenzen *et al.*, 2001), utilize components of the transport machinery for their nuclear targeting. Our studies demonstrate that Sgk contains a functional NLS that is recognized by importin- $\alpha$ . The efficient import of Sgk requires the presence of an energy source and an intact NPC in the digitonin permeabilized nuclear import system. This result demonstrates the involvement of components of the active transport machinery, generally involving the importin- $\alpha/\beta$  heterodimer.

Strikingly, addition of a carboxy-terminal fragment of importin- $\alpha$  (which retains Sgk binding but lacks an importin- $\beta$  binding domain) in the in vitro nuclear import assay system, profoundly inhibited the efficiency of Sgk import. Presumably, the dominant negative effects of this importin- $\alpha$  fragment result from competition with endogenous importin- $\alpha$  for binding to Sgk, directly implicating importin- $\alpha$  in the nuclear import of Sgk. It is well established that the importin- $\alpha$  receptor dependent-pathway mediates nuclear targeting several transcription factors such as p53, Stat-1, glucocorticoid receptor (Gorlich and Mattaj, 1996; Ferrigno *et al.*, 1998; Gorlich, 1998; Truant *et al.*, 1998; Gamblin and Smerdon, 1999; Christophe *et al.*, 2000; Jans *et al.*, 2000; Sweitzer *et al.*, 2000), and our study represents the first report to establish a role for the importin- $\alpha$  in the serum induced, active transport of a serine/threonine protein kinase, Sgk. The nuclear function of Sgk is not readily apparent as knowledge on the nuclear substrates of Sgk is limited. Recent studies have identified the FKHRL1 protein, a forkhead transcription factor family

member as a Sgk substrate (Brunet *et al.*, 2001), although it is unclear whether this phosphorylation event occurs in the nuclear compartment.

Functional analysis of the NLS identified the central catalytic domain demonstrated that the lysine residues within the KKAILKKKEEK sequence were critical for importin- $\alpha$  binding in vitro, and for serum dependent nuclear translocation in intact cells (see Figure 8). Typically, NLS motifs can be distinguished into the monopartite type enriched in a single cluster of basic amino acids (PKKKRKV) and exemplified by the SV40 large T antigen, or the bipartite class, initially identified in the nucleoplasmin protein which consists of two clusters of basic amino acids separated by a variable spacer (KRPAATKKAGQAKKKK) (Robbins *et al.*, 1991). The Sgk NLS (KKAILKKKEEK), conforms to the consensus bipartite NLS (KK(10-12)KKK), containing a short spacer flanked by two clusters of lysines. Our mutational analysis, did not define the specific lysines or cluster of lysines crucial for Sgk- importin- $\alpha$  interactions and nuclear targeting, as all the six lysines within the sequence KKAILKKKEEK were altered to alanines. Conceivably, only one set of lysines or both may be essential for the serum induced nuclear transport of Sgk, although in numerous studies, both clusters of basic amino acids have been determined to be essential in a wide spectrum of NLS containing import substrates for efficient nuclear targeting (Bryan and Morasso, 2001; Djabali *et al.*, 2001; Hood *et al.*, 2001; Qiu *et al.*, 2001). Studies are in progress to identify the specific sets of lysines within the Sgk NLS, that are important for importin- $\alpha$  dependent interactions and regulated nuclear entry. It is also worth noting that upon aligning the sequences of the

three Sgk isoforms, there are NLS-like sequences in Sgk2 (KKSILKNKEQN) and Sgk3 (KKIVLNRKEQK), which are generally similar to the KKAILKKKEEK sequence in Sgk1, that have not been functionally tested.

Generally, binding of importin- $\alpha$  to the NLS involves two sites within the import receptor molecule, a major site spanning the amino-terminal end of the receptor between the first and fourth arm repeats, and a minor site located at the carboxy -terminus between arm repeats 4 and 8 (Conti and Kuriyan, 2000; Fontes *et al.*, 2000). However in our studies, only half of the eighth repeat through the carboxy -terminal end of importin- $\alpha$  was sufficient for binding to Sgk when expressed as a bacterial GST fusion partner, suggesting that the proximal arm repeats of importin- $\alpha$  may be dispensable for binding as has been reported in some cases (Bannister *et al.*, 2000; Kovac *et al.*, 2000 ; Hieda *et al.*, 2001). We have been unable to confirm whether the carboxy tail of importin- $\alpha$  when expressed as a truncated protein in cells retains its ability to associate with Sgk *in vivo*, as our attempts to express truncated importin- $\alpha$  protein in mammalian cells have been unsuccessful, perhaps due to the intrinsic instability of such structurally altered proteins. However, because the functional NLS within Sgk (KKAILKKKEEK) resembles the bipartite NLS consensus sequence, one prediction is that, both the minor and major sites within the importin- $\alpha$  molecule spanning the arm repeats may be required for functional interactions between Sgk- importin- $\alpha$  *in vivo* and warrants further studies. Furthermore, based on the Sgk homology to other kinase catalytic domains, a Swiss Model 3-D threading program was

used to theoretically show that the Sgk NLS is located on the surface of its catalytic domain, and therefore likely accessible to importin- $\alpha$ .

Several studies have advanced the notion that phosphorylation induced homodimerization of import substrates plays a critical role in nuclear-cytoplasmic shuttling of protein cargos (Yoneda, 2000; Cyert, 2001; Nagoshi and Yoneda, 2001). Conversely, phosphorylation of specific sequences in the vicinity of the NLS can either enhance (e.g. SV40 antigen) (Rihs *et al.*, 1991) or attenuate (Brownawell *et al.*, 2001) the efficiency of nuclear localization, demonstrating the influence of phosphorylation dependent regulatory modules on NLS-driven nuclear localization. We have observed that the hypophosphorylated forms of Sgk interact with importin- $\alpha$  in vitro; however the serum induced nuclear transport of either endogenous Sgk or exogenously expressed Sgk mutants is abrogated under conditions that ablate Sgk phosphorylation. These observations show that the PI3-kinase dependent phosphorylation of Sgk is vital for its nuclear targeting. Our studies further show that Sgk kinase activity is not a molecular requirement for either efficient importin- $\alpha$  binding or for nuclear targeting. In agreement with our findings, stimulus specific nuclear import of Erk2/MAPK and p38/MAPK was shown to be sensitive to phosphorylation dependent activation but independent of their kinase activities (Gaits *et al.*, 1998; Lenormand *et al.*, 1998; Brunet *et al.*, 1999; Hulleman *et al.*, 1999). Evidently, activation of these translocating kinases via phosphorylation enhances their dimerization function, a property construed to be critical for their efficient

nuclear translocation (Khokhlatchev *et al.*, 1998). Extensive dimerization of the Akt/PKB kinase, which is highly homologous to Sgk, has been documented (Coffer *et al.*, 1998), suggesting a similar mode of regulation for Sgk protein activation and nuclear transport. Collectively, these data demonstrate that serum induced nuclear translocation of Sgk is dictated by two key structural attributes within Sgk, that consists of the Sgk NLS, and the phosphorylation status of Sgk. We are currently exploring the functional ramifications of disruptions in Sgk-importin- $\alpha$  interactions on the stimulus dependent cell signaling events.

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Abreviations:

Sgk, serum and glucocorticoid inducible protein kinase; PI 3-kinase, phosphatidylinositol kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PBS, phosphate buffered saline, p38/MAPK, 38 kDa mitogen activated protein kinase; Akt/PKB, Akt/protein kinase B; PKC, protein kinase C; PKA, protein kinase A; SP1, SV40 early promoter transcription factor-1, Erk/MAPK, Extracellular regulated protein kinase/Mitogen activated protein kinase; NLS, nuclear localization signal; NPC, nuclear pore complex; GFP, green fluorescence protein, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

## FIGURE LEGENDS

**Figure 1.** Identification of importin- $\alpha$ -1 as a Sgk interacting protein by yeast two hybrid assay. (A) The yeast host strain EGY48 containing the Leu+ and LacZ reporter plasmids were transformed with the library derived importin- $\alpha$  clones 1, 2, 3 along with one of three bait plasmids, pLexA-Sgk bait (top panel), pLexA-Lamin (middle panel), or pLexA-Bicoid (lower panel). Individual Ura+ His+ Trp+ transformants were streaked onto synthetic plates containing galactose-raffinose based drop out media, lacking uracil, histidine, tryptophan and leucine. The plates were incubated at 30°C for 3 days and ability of individual bait proteins to interact with the library derived importin- $\alpha$  was determined by the growth of yeast on the selective medium. Similar results were obtained in two separate experiments. (B) The structure diagram of 529 amino acid full length mouse importin- $\alpha$  (imp $\alpha$ ) depicts the N-terminal importin- $\beta$  binding domain, 8-9 arm repeats traversing the central domain and the carboxy -terminal enriched in acidic amino acids. The alignment of the truncated importin-  $\alpha$  ( $\Delta$ 1-422) clone 1 isolated from the yeast two hybrid screen is shown in comparison to the full length importin- $\alpha$  (imp $\alpha$ ). The library derived importin- $\alpha$  is an N-terminal truncation that begins at amino acid 423 and continues to the C-terminus at amino acid 529. Each of the three library derived clones displayed identical sequence homology and the size of encoded truncated protein only differed by 3-4 amino acids at the N-terminal truncation.

**Figure 2.** Analysis of Sgk and importin- $\alpha$  interactions by in vitro GST pull down assays.

(A) Full length Sgk cDNA was transcribed and translated in vitro with a rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine to produce [ $^{35}$ S]Sgk. The SDS-PAGE and autoradiographic analysis of the unprogrammed lysate (UL) devoid of any Sgk cDNA is shown in the far left lane, and the programmed lysate representing an aliquot of the translated protein used for the binding assays is shown in the input lane. The in vitro translated [ $^{35}$ S]Sgk was incubated with glutathione sepharose beads bound with GST alone (GST), a fusion protein linked to the truncated importin- $\alpha$  (GST- $\Delta$ 1-422), or with a GST-fusion protein linked to the full length importin- $\alpha$  (GST-imp $\alpha$ ). Proteins retained on the beads were resolved by SDS-PAGE and visualized by autoradiography. The electrophoretic migration of the molecular weight standards in kDa are also shown. (B) GST pull down assays using the GST-full length importin- $\alpha$  fusion protein (GST-imp $\alpha$ ) were carried out with in vitro translated [ $^{35}$ S]Sgk, [ $^{35}$ S]Jnk, or [ $^{35}$ S]PKC $\zeta$  and the proteins retained on the glutathione sepharose beads analyzed by SDS-PAGE and autoradiography. The in vitro translated protein used in the assay is shown in the input lanes. (C) Subconfluent cultures of Con8.hd6 mammary epithelial tumor cells were serum starved for 72 hours and then pulsed with 10% calf serum containing DME/F12 media for 4 hours (+S), whereas control cultures were continued on serum free media alone (-S). Cell extracts from serum stimulated (+S) were incubated with either GST alone, with the full length importin- $\alpha$  fusion protein GST-imp $\alpha$  (left panel), or the truncated importin- $\alpha$  fusion protein GST- $\Delta$ 1-422 (right panel) bound to glutathione sepharose beads. The bound proteins were separated on SDS-PAGE and immunoblotted with anti-Sgk antibodies to

detect binding of the endogenous Sgk. As indicated in each panel, the input lanes show 10% of the cell lysate containing the endogenous Sgk included in the assay. Extracts from uninduced samples (-) are shown in the left panel. Arrows indicate migration of Sgk protein and the electrophoretic migration of molecular weight marker proteins are shown on the left side of each panel. Similar results were obtained in multiple experiments.

**Figure 3.** Co-immunoprecipitation of Sgk and importin- $\alpha$ . (A) Con8.hd6 mammary epithelial tumor cells were transfected with HA-importin- $\alpha$  expression vector (full length importin- $\alpha$ ) using lipofectamine method, and following serum starvation for 36 hours, cells were stimulated for 4 hours with 10% calf serum containing media to induce the production of endogenous Sgk. Precleared cell lysates were prepared as described in the Methods section, immunoprecipitated with anti-Sgk antibodies (+) or with control rabbit IgG antibodies (-), and protein bound to the protein-A beads fractionated by SDS-PAGE and immunoblotted with anti-HA monoclonal antibodies (right panel). Inputs (10%) reveal expression of HA-importin- $\alpha$  in the transfected samples compared with vector control lysates. The efficiency of Sgk immunoprecipitations were determined by immunoblotting the precleared lysates before (Pre) and after (Post) the Sgk-immunoprecipitation with anti-Sgk antibodies (left panel). (B) Hek 293 cells were transfected with expression plasmids encoding full length importin- $\alpha$  (HA-importin- $\alpha$ ) and the catalytic domain of Sgk (Cat Sgk), or with the empty vector controls. The cell lysates were prepared as detailed in Methods section, and immunoprecipitated with anti-HA monoclonal antibodies (+) or with control

mouse IgG antibodies (-), and Sgk bound to the protein-G beads detected with anti-Sgk immunoblotting (right panel). Inputs (HA-imp $\alpha$  and Cat Sgk) contain 10% of total protein and are compared to lysates prepared from vector transfected controls as indicated in each panel. Anti-HA immunoblotting of supernatants before (Pre) and after (Post) immunoprecipitating with anti-HA antibodies, to determine efficiency of immunoprecipitation of the HA-imp $\alpha$  is shown in the left panel. The electrophoretic migration of molecular weight marker proteins is shown on the left side of each panel and arrows indicate migration of HA-imp $\alpha$  and heavy chain. Similar results were obtained from three independent experiments.

**Figure 4.** Subcellular localization of Sgk and importin- $\alpha$ . (A) Low confluent monolayers of Con8.hd6 mammary epithelial tumor cells grown on 2 well lab-tek slides were transfected with expression vectors encoding the full length HA-tagged importin- $\alpha$  (HA-imp $\alpha$ ) using lipofectamine. Cells were serum starved for 36 hours, and then serum boosted with 10% calf serum (+S) for 15 hours. One set of cells were maintained under serum free conditions for the duration of the experiment (-S). The co-localization of Sgk and transfected HA-importin- $\alpha$  was examined by double indirect immunofluorescence microscopy using anti-Sgk polyclonal antibodies and anti-HA monoclonal (1:1000, dilution) antibodies. The secondary antibodies used to detect the Sgk immune reaction was fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies, while, Texas-red/goat anti-mouse antibodies was used to detect the HA-importin- $\alpha$ . Protein expression

of endogenous Sgk and exogenously expressed HA-importin- $\alpha$  in the treatment conditions used for co-localization studies were evaluated by immunoblotting with anti-Sgk or anti-HA antibodies respectively. The electrophoretic migration of molecular weight marker proteins are shown on the left side of each gel and arrows indicate migration of Sgk and HA-imp $\alpha$ . (B) Low confluent monolayers of Con8.hd6 mammary epithelial tumor cells grown on 2 well lab-tek slides were transfected with an expression vector encoding the Green Fluorescence Protein-tagged Sgk (GFP- Sgk) fusion protein. Cells were serum starved for 36 hours, and then serum boosted with 10% calf serum (+S) for 15 hours. One set of cells were maintained under serum free conditions for the duration of the experiment (-S). The localization of GFP-Sgk was monitored by direct fluorescence microscopy of formaldehyde fixed cells using the FITC filter as detailed in Methods Section. Protein expression of endogenous Sgk and exogenously expressed GFP-Sgk in the treatment conditions used for co-localization studies were evaluated by immunoblotting with anti-Sgk.

**Figure 5.** Characteristics of the in vitro nuclear import of HA-Sgk. Hek 293 cells were transfected with expression vectors encoding the HA-Sgk, and S-100 cell cytosols prepared as described in the Methods section. Nuclear import of the exogenously expressed Sgk proteins was examined in vitro either in the absence (-ATP) or presence of (+ATP) regenerating system (upper panel), or in the absence (-) or presence (+) of cytosolic extract (middle panel), or with cytosol in the absence (-) or presence (+) of 0.5 mg/ml wheat germ agglutinin (lower panel) using nuclei derived from permeabilized Hela cells. The import

of HA-Sgk was assessed by indirect immunofluorescence microscopy using anti-HA antibodies as detailed in the text. (B) The lower panel shows expression of exogenously derived HA-Sgk in S-100 cytosols from Hek 293 cells as determined by anti-HA immunoblotting.

**Figure 6.** Carboxyterminal fragment of importin- $\alpha$  acts as a dominant negative molecule that inhibits in vitro Sgk nuclear import. Nuclear Import reactions comprised of an intact ATP regenerating system, and cytosols containing either ectopically expressed HA-Sgk or serum activated endogenous Sgk, were carried out in the presence of purified GST alone or GST- $\Delta$ 1-422 (4  $\mu$ g each) and entry of the import substrates into permeabilized Hela nuclei assessed by indirect immunofluorescence using anti-HA antibodies for HA-Sgk or polyclonal Sgk antibodies for endogenous Sgk.

**Figure 7.** Interaction of in vitro translated Sgk mutants with recombinant full length importin- $\alpha$ . (A) The structural diagrams are shown for the wild type Sgk (Wt Sgk), the kinase dead mutant Sgk (K127M) Sgk, an N-terminal deleted Sgk ( $\bullet$ N 61-431 Sgk), a C-terminal deleted Sgk ( $\bullet$ C 1-355 Sgk), a double truncation representing the central catalytic domain only of Sgk (Cat 60-355 Sgk), or two fragments of the central domain of Sgk (60-157 Sgk, 60-122 Sgk). (B) The [ $^{35}$ S]methionine-labeled forms of the wild type and indicated mutant Sgk proteins were synthesized using an in vitro translation system as described in Figure 2 and the resulting proteins (IVT inputs, left panel) analyzed by SDS-PAGE and autoradiography. The in vitro translated Sgk proteins (right panel) were

incubated with either the GST protein alone or the GST full length importin- $\alpha$  fusion protein (GST-imp $\alpha$ ) bound to glutathione sepharose beads, and the bound proteins were separated on SDS-PAGE and visualized by autoradiography. (C) GST pull down assays using the indicated fragments of the Sgk central catalytic domain were performed as above, except with the inclusion of truncated importin- $\alpha$  (GST- $\Delta$ 1-422), in addition to the full length importin- $\alpha$  (GST-imp $\alpha$ ) and GST alone. The electrophoretic migration of molecular weight markers are shown on the left side of each panel. Similar results were observed in three independent experiments.

**Figure 8.** Identification of the Sgk NLS required for binding to importin- $\alpha$  and serum induced nuclear import. (A) Site directed mutagenesis of the Sgk NLS. The Sgk NLS mutant was generated by substitution of the six highlighted lysines with alanines within the context of the full length Sgk and is shown in comparison with the consensus bipartite NLS. (B) In vitro translated wild type Sgk (Wt [ $^{35}$ S]Sgk ) and NLS mutant Sgk (NLS Mut [ $^{35}$ S]Sgk ) proteins were incubated with either the GST protein alone or the GST-full length importin- $\alpha$  fusion protein (GST-IMPA) bound to glutathione sepharose beads, and the bound proteins were separated on SDS-PAGE and visualized by autoradiography (left panel). Cell lysates prepared from Hek 293 cells transiently transfected with expression plasmids encoding HA-tagged forms of wild type Sgk (HA-Wt Sgk ) or NLS mutant Sgk (HA-NLS Mut Sgk) were incubated with GST protein alone or with the full length importin- $\alpha$  fusion protein GST-IMPA bound to glutathione sepharose beads, and the proteins retained on the beads fractionated by SDS-PAGE and immunoblotted with anti-

HA antibodies (right panel). Inputs denote 10% of the total protein included in the binding reaction, and the electrophoretic migration of molecular weight marker proteins are shown on the left side of each panel. (C) Low confluent monolayers of Con8.hd6 mammary epithelial tumor cells grown on 2 well lab-tek slides were transfected with expression vectors encoding either the wild type HA-tagged Sgk (HA-Wt Sgk) or the NLS mutant Sgk (HA-NLS Mut Sgk) as in Figure 4. Following serum starvation for 36 hours, cells were serum boosted with 10% calf serum (+S) for 15 hours whereas the control slides remained on serum free media (-S). The subcellular distribution of the wild type and NLS mutant Sgk proteins were evaluated by indirect immunofluorescence microscopy using anti-HA monoclonal (1:1000, dilution) antibodies. Texas-red/goat anti-mouse antibodies was used as the secondary antibodies as in Figure 4 for detection of the HA-tagged proteins.

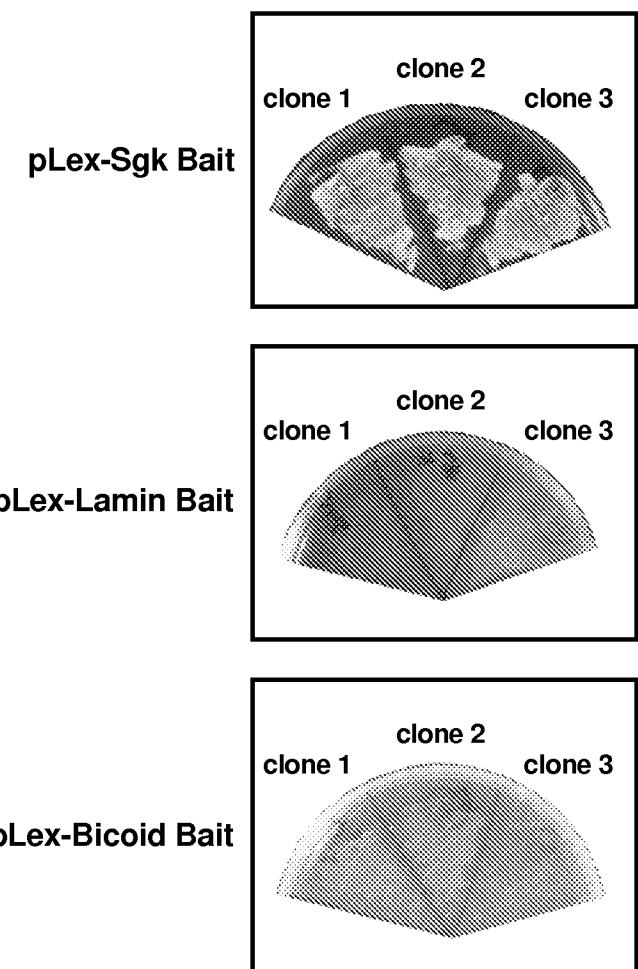
**Figure 9.** Characterization of Sgk phosphorylation and kinase mutants in the in vitro interaction with importin- $\alpha$  and in serum induced nuclear translocation. (A) Hek 293 cells were transiently transfected with expression plasmids encoding HA-tagged forms of wild type Sgk (HA-Wt Sgk), kinase dead Sgk (HA-K127M Sgk), double phosphorylation site mutant of Sgk (HA- Sgk T256A/S422A), or with an empty expression vector (vector control) as described in the text. Cell lysates were incubated with GST protein alone or with the full length importin- $\alpha$  fusion protein GST-imp $\alpha$  bound to glutathione sepharose beads, and the proteins retained on the beads fractionated by SDS-PAGE and immunoblotted with anti-HA antibodies (left panels) as described in Figure 8. Inputs

denote 10% of the total protein included in the binding reaction and are compared with vector transfected controls. (B) Low confluent monolayers of Con8.hd6 mammary epithelial tumor cells were transfected with wild type Sgk (HA-Wt Sgk), kinase dead Sgk (HA-K127M Sgk), or with single phosphorylation site mutants (HA-T256A Sgk, HA-S422A Sgk) or a double phosphorylation site mutant (HA-T256A/S422A SGK) of Sgk. All constructs contained a HA epitope tag. Cells were serum starved for 36 hours, and then serum boosted with either 10% calf serum (+ S) for 15 hours or continued on serum free media (- S). The subcellular distribution of the Sgk proteins were assessed by indirect immunofluorescence microscopy using anti-HA monoclonal antibodies (right panels) as described in Figure 8.

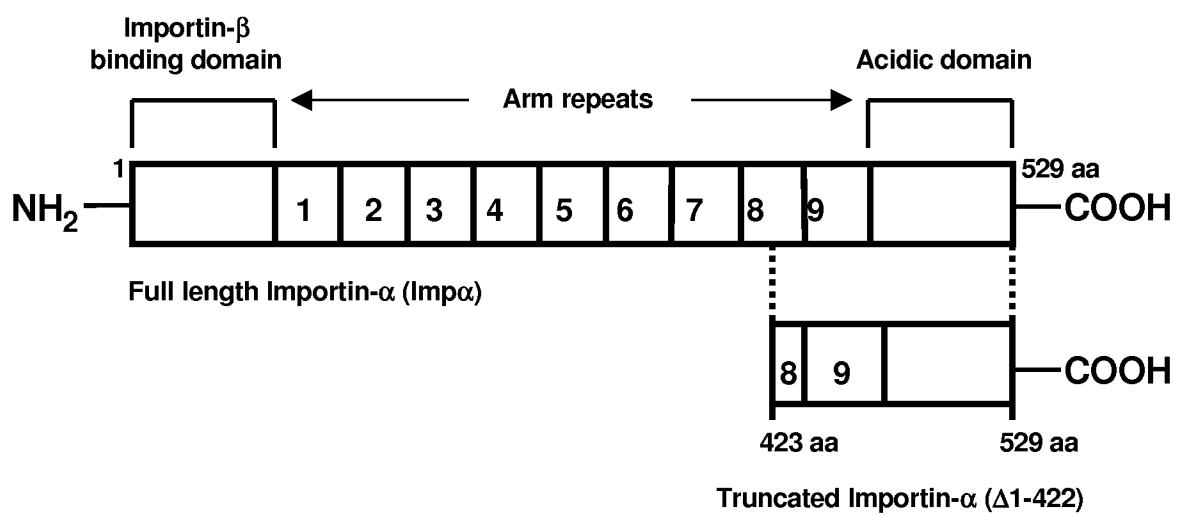
**Figure 10.** Effects of altering the PI 3-kinase dependent phosphorylation of Sgk on the in vitro interaction with importin- $\alpha$  and in serum induced nuclear translocation. (A) Subconfluent cultures of Con8.hd6 mammary epithelial cells were serum starved for 48 hours and pretreated with or without 50  $\mu$ M LY294002, a PI3-Kinase inhibitor, for 12 hours. Cells were stimulated for 4 hours with 10% calf-serum containing DMEM/F12 media either in the absence or presence of LY294002. Cell extracts from LY294002 treated (+) or untreated (-) cells were prepared as described in the Methods section, incubated with GST protein alone or with the full length importin- $\alpha$  fusion protein GST-Imp $\alpha$  bound to glutathione sepharose beads, and the proteins retained on the beads fractionated by SDS-PAGE and immunoblotted with anti-Sgk antibodies. Input lanes depict 10% of the extract prepared from LY294002 treated or untreated samples used in the binding assay

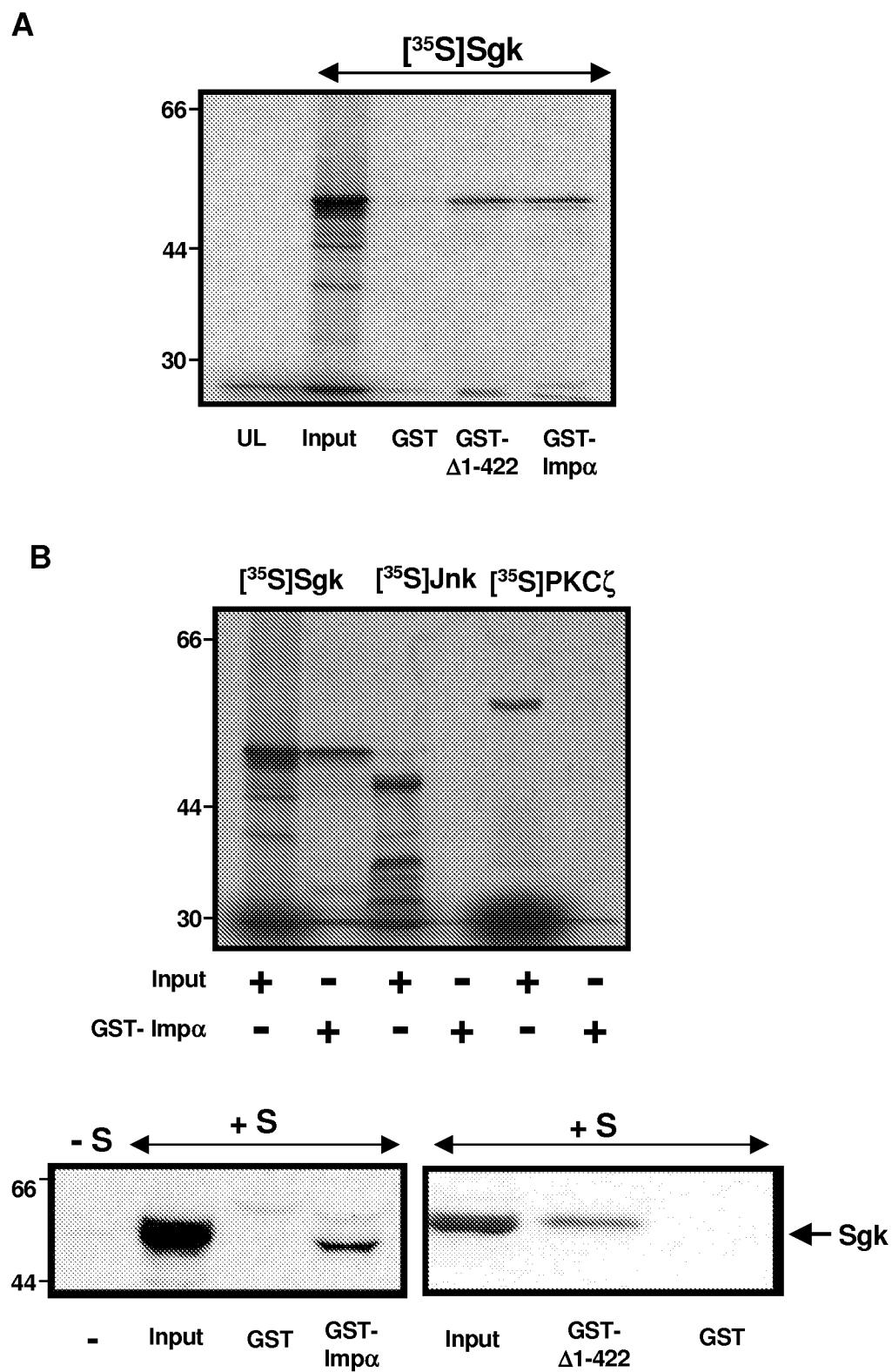
(upper panel). (B) The nuclear localization of endogenous Sgk or exogenously expressed mutated Sgk with the T256 and S422 phosphorylation sites converted into aspartic acid (HA-T256D/S422D Sgk) and containing an HA epitope tag was assessed in Con8.hd6 mammary epithelial tumor cells. Low confluent monolayers of transfected and nontransfected cells were serum starved for 36 hours, and then serum boosted with 10% calf serum (+ Serum) for 15 hours, or maintained under the serum free conditions (- Serum). Other sets of cells were pretreated with 50  $\mu$ M LY294002 for 8 hours, and subsequently incubated with serum in the continued presence of LY294002 for another 15 hours (+Serum +LY). The localization of endogenous Sgk was examined by indirect immunofluorescence microscopy using anti-Sgk polyclonal antibodies (lower panel) as described in Figure 4.

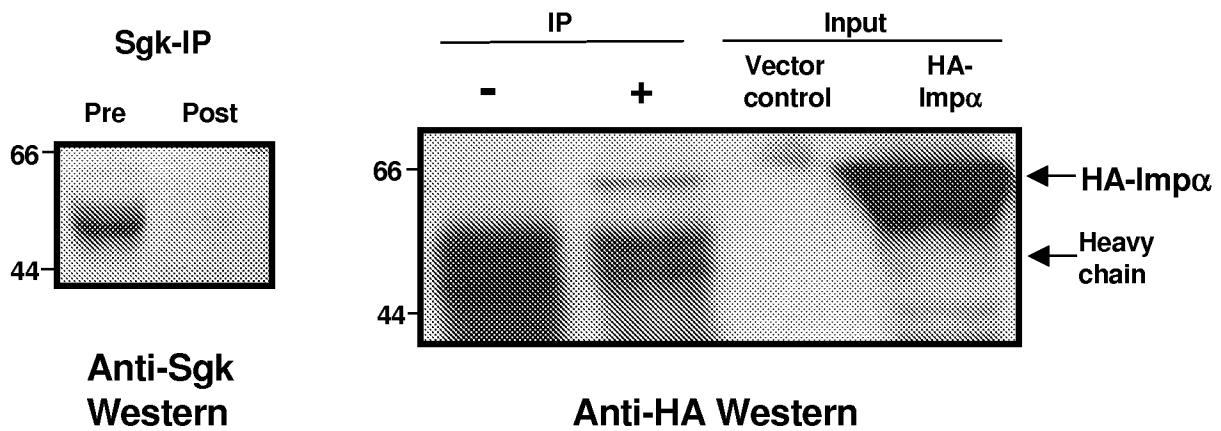
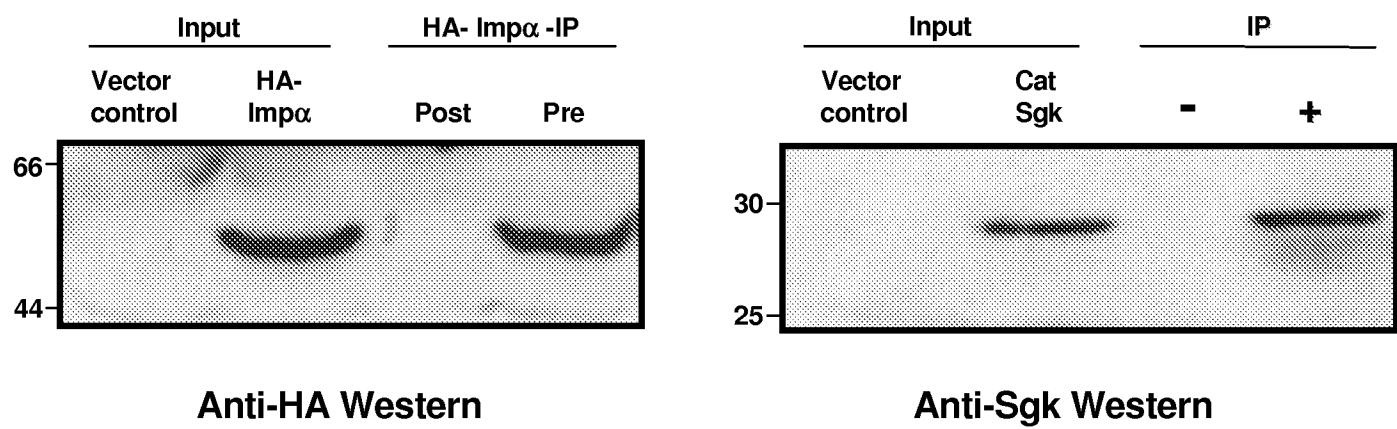
**A**

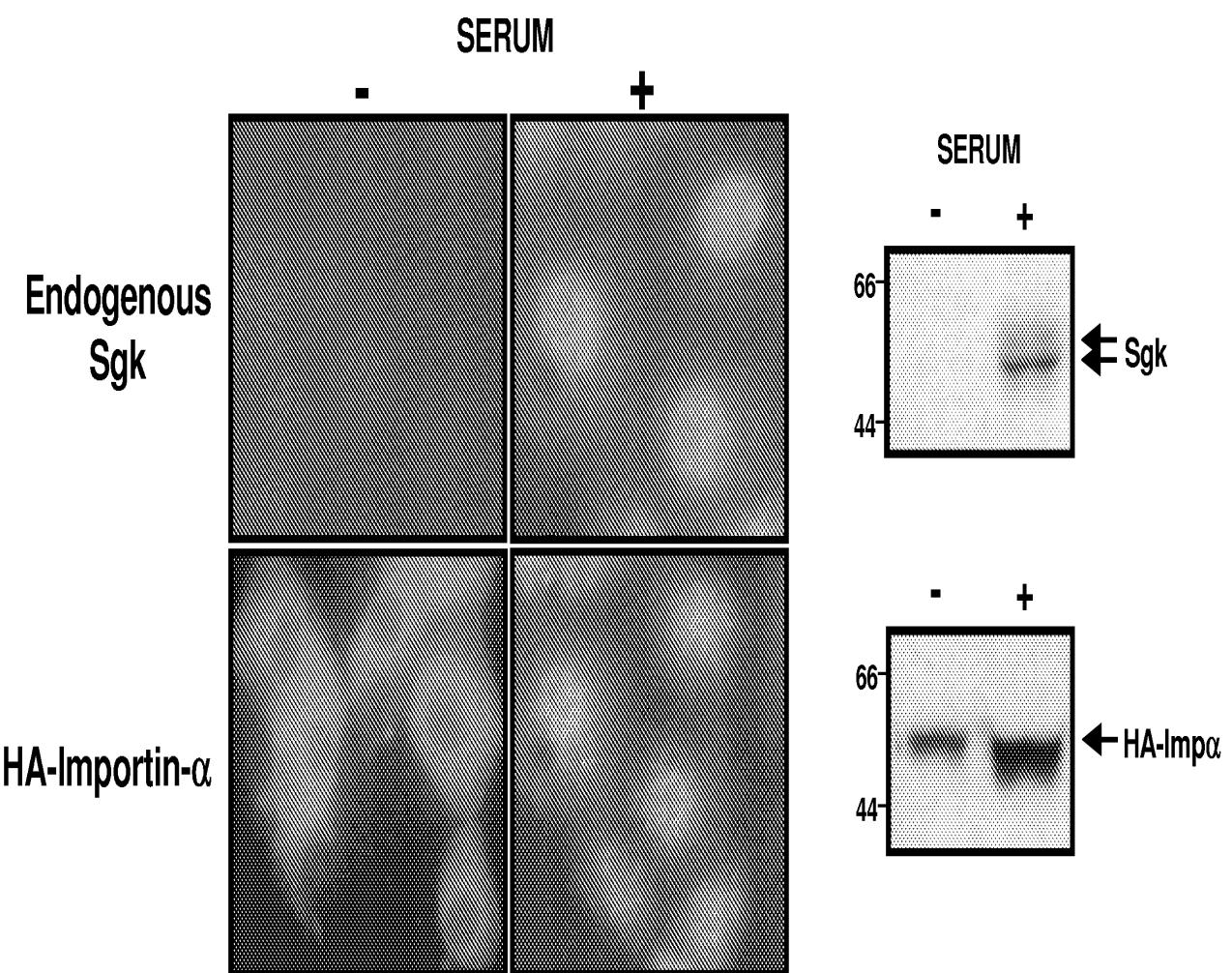
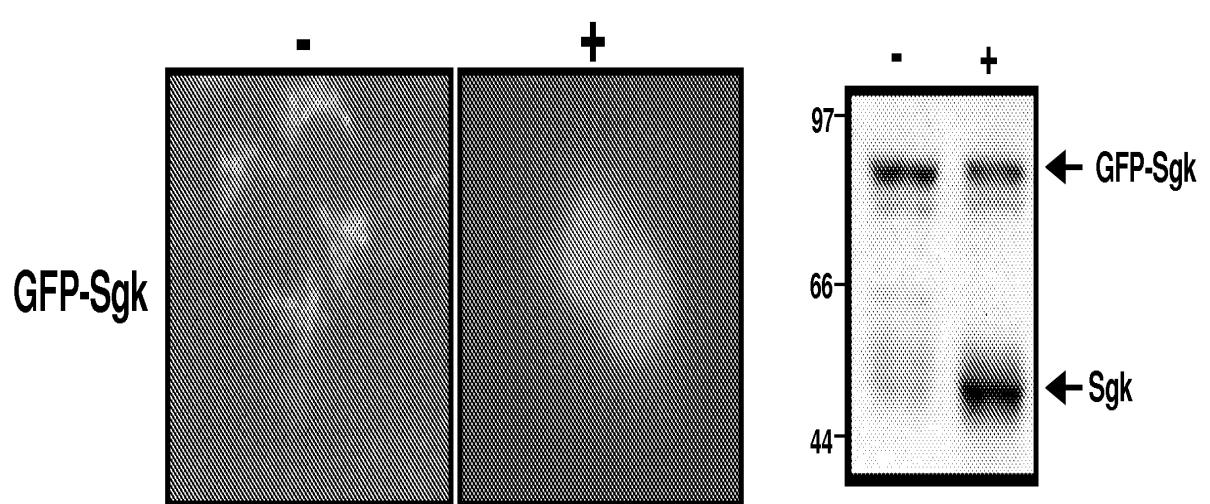


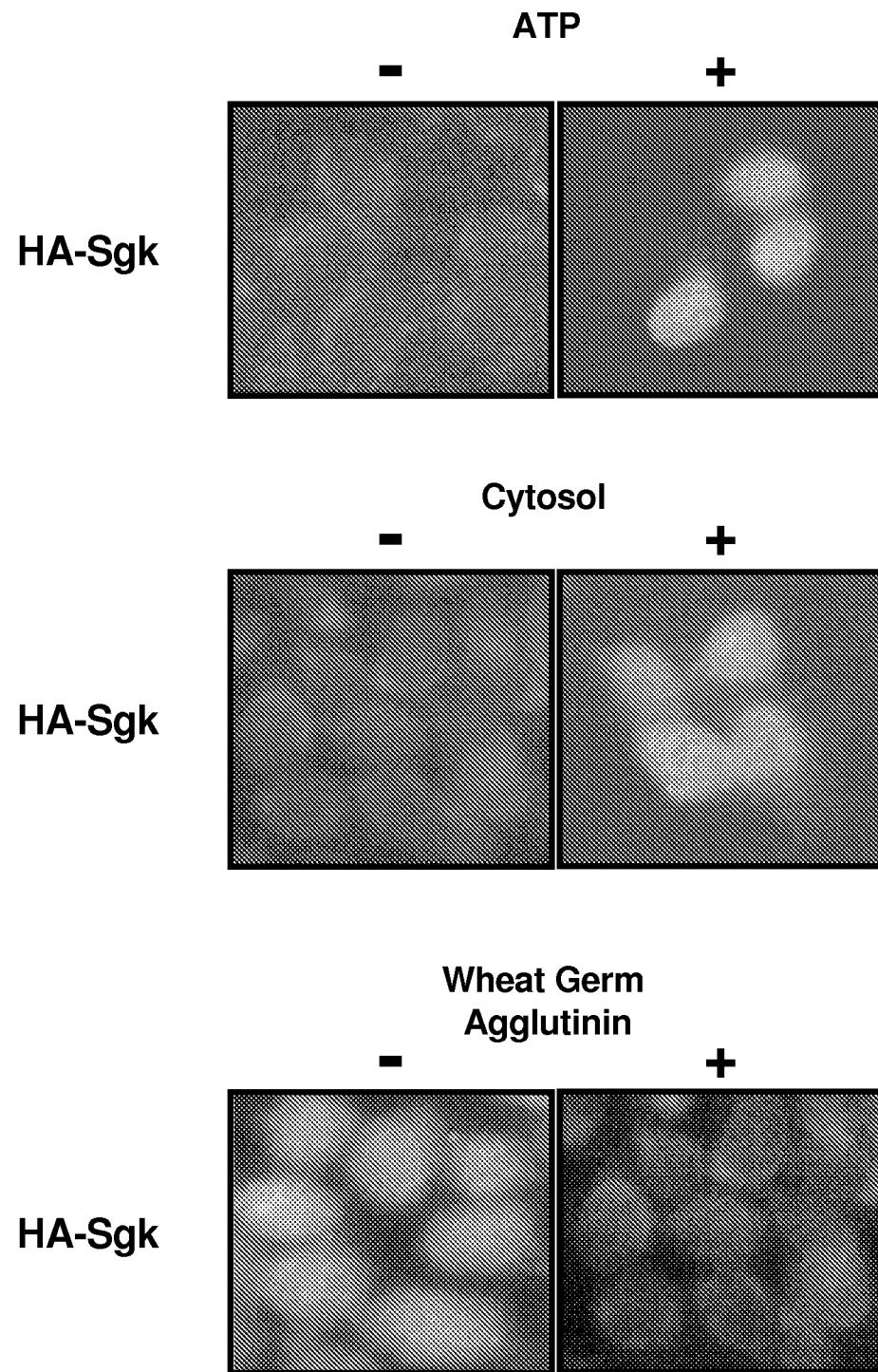
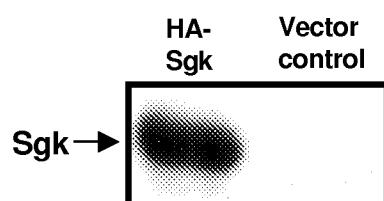
**B**

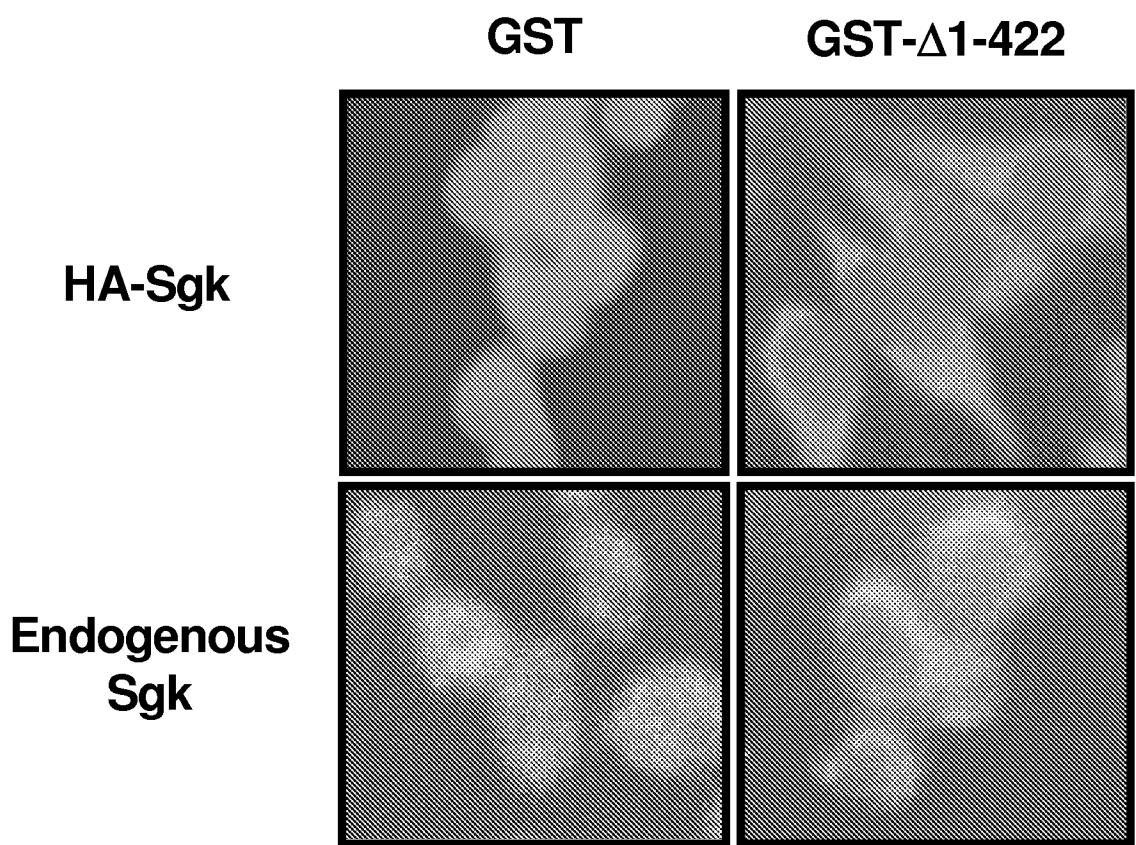


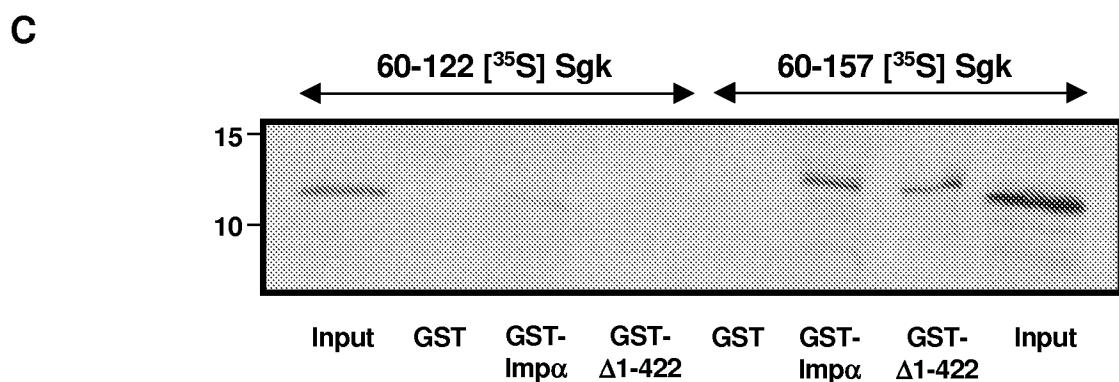
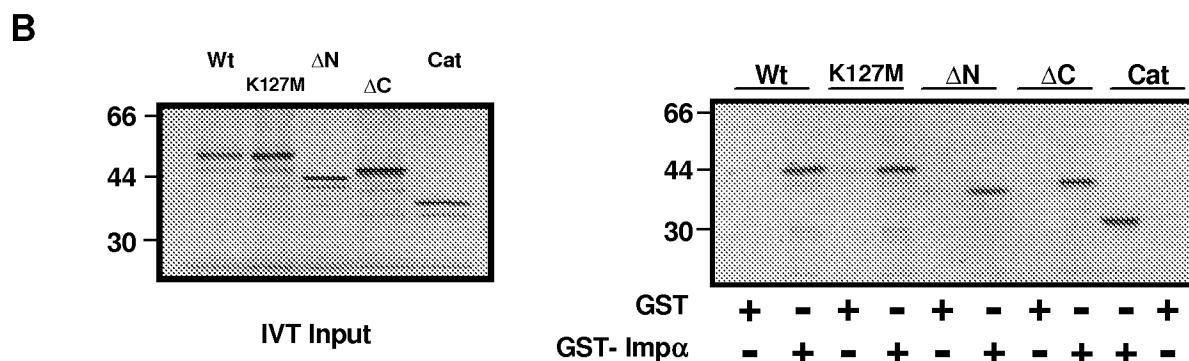
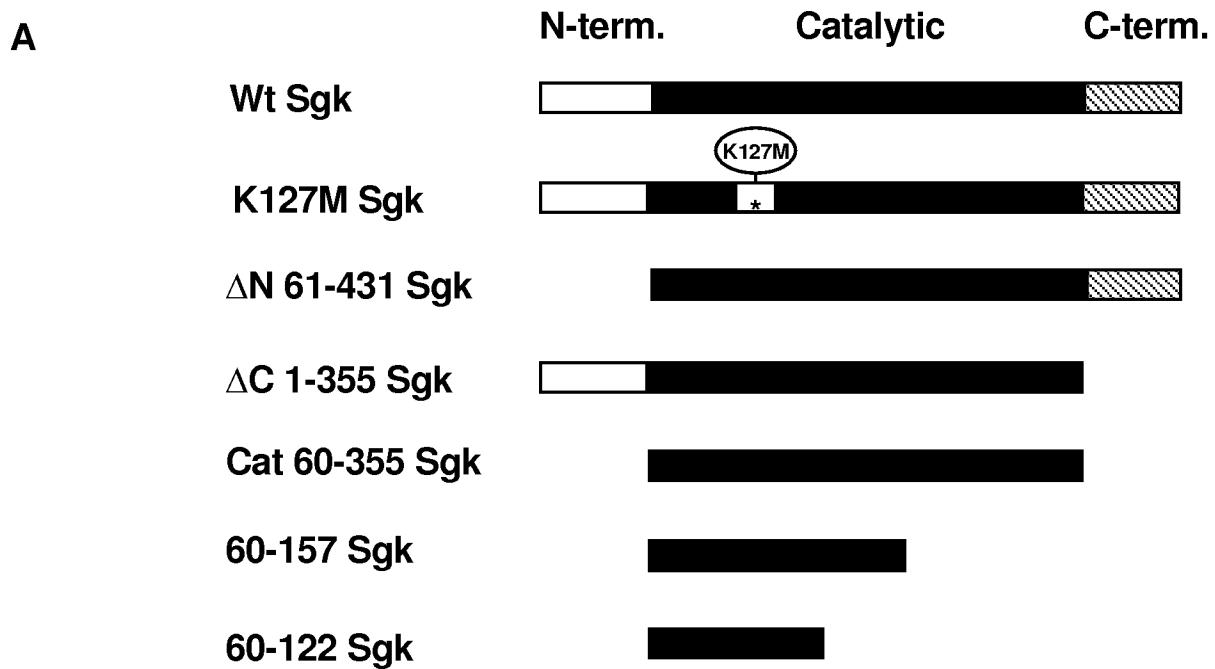


**A****Sgk-IP/ HA-Imp $\alpha$  Western****B****HA-Imp $\alpha$ -IP/ Sgk Western**

**A****B**

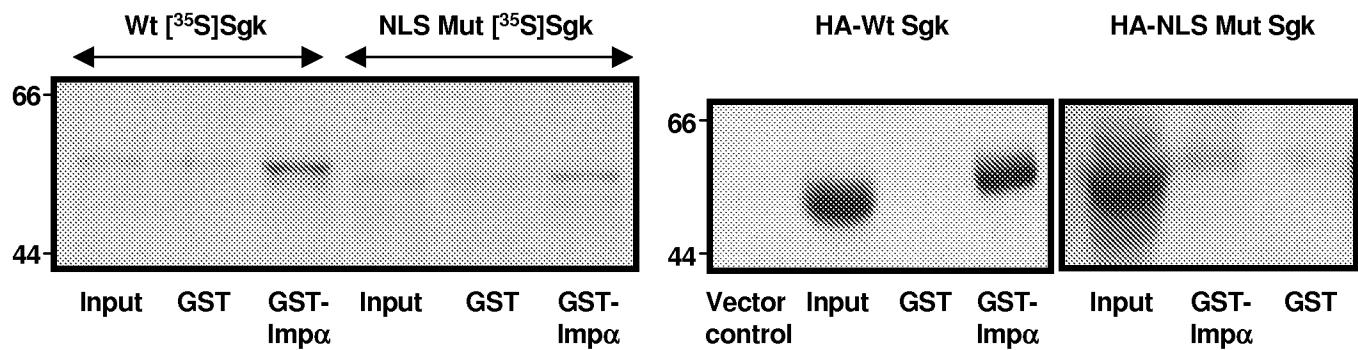
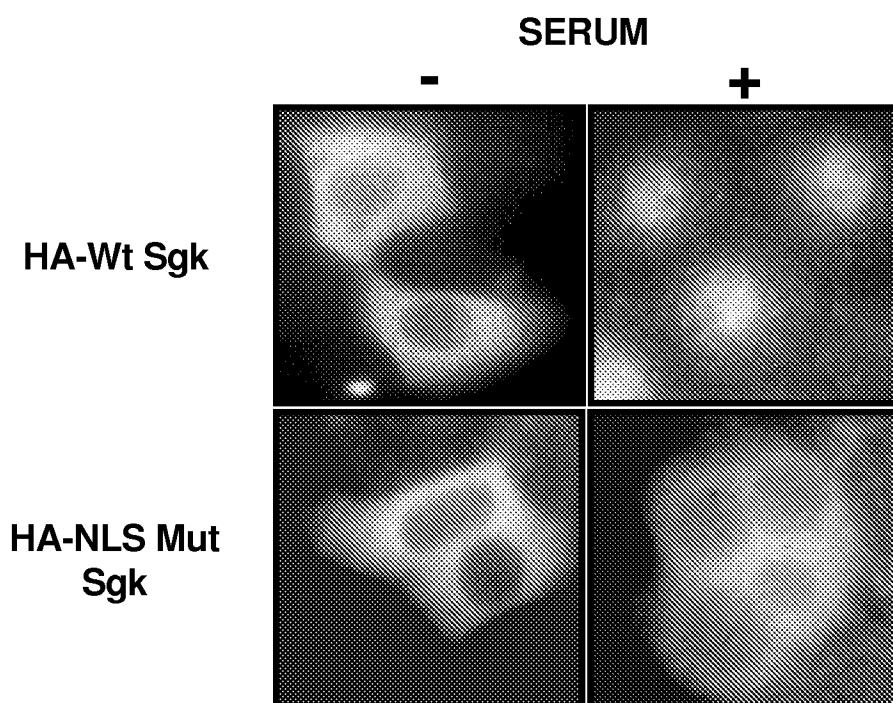
**A****B**



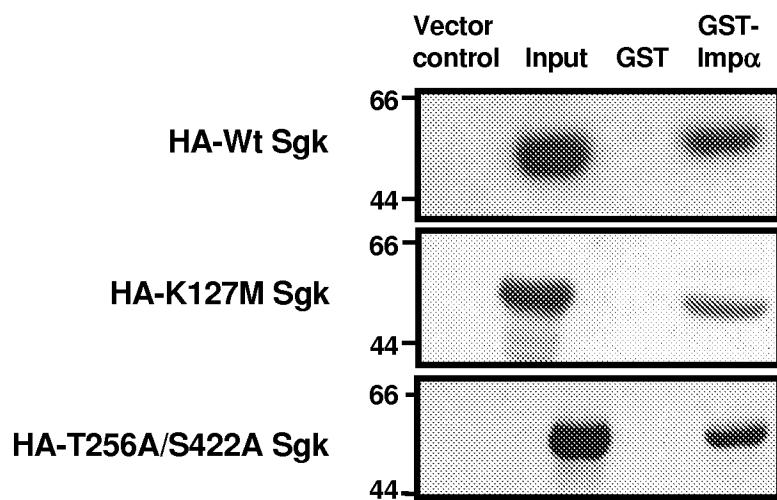


**A**

**Sgk NLS**  
131                    141  
**Wt Sgk**            KKAILKKKEEK  
**NLS Mut Sgk**      AAAILAAAEEA  
**Bipartite NLS consensus**    KK(10-12aa)KKK

**B****C**

**A**



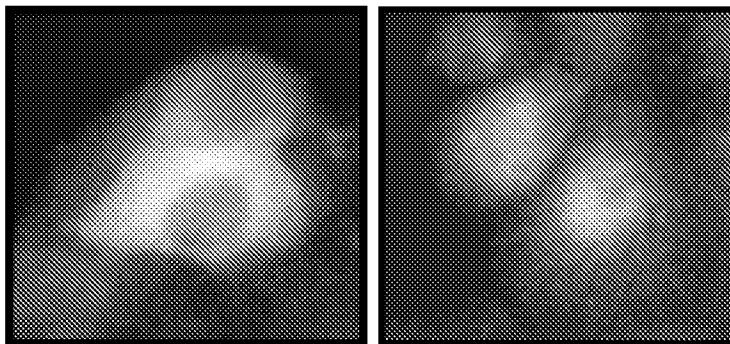
B

SERUM

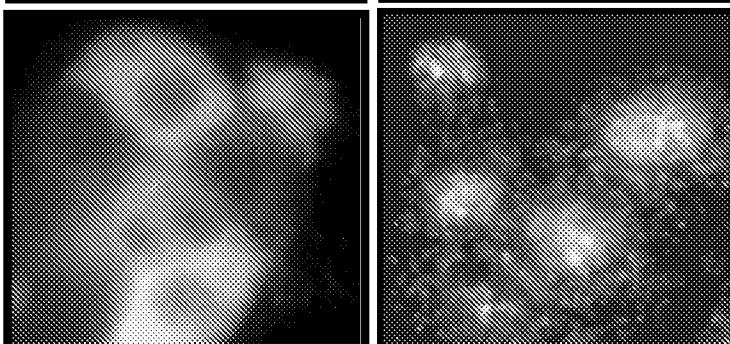
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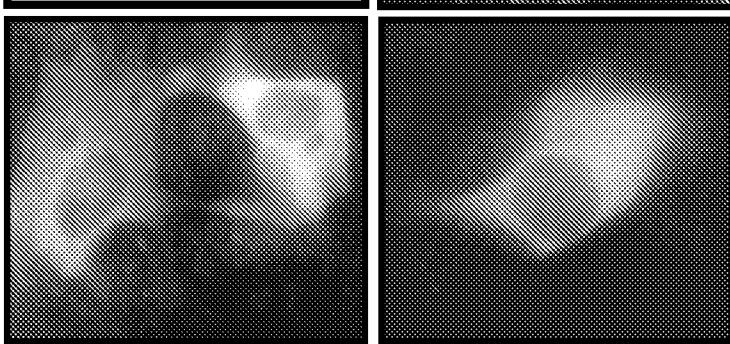
HA-Wt Sgk



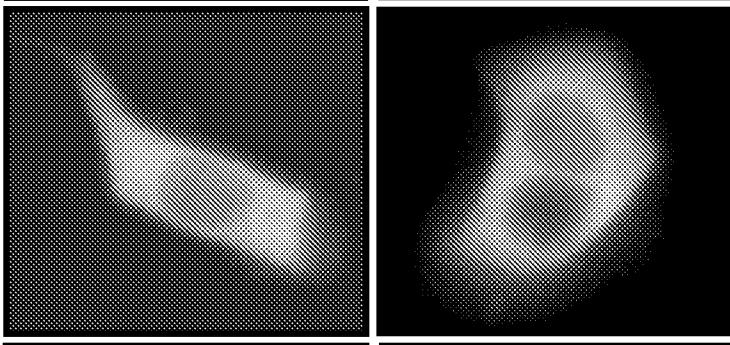
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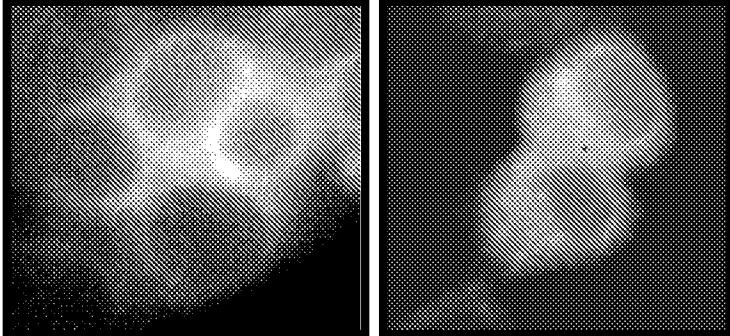
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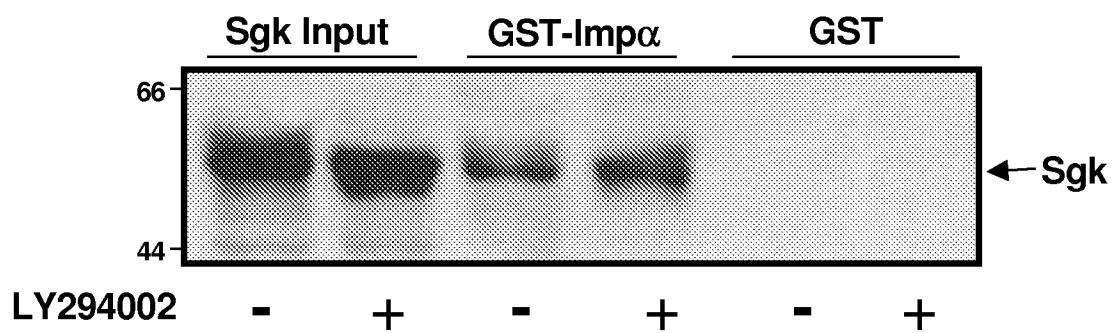
HA-S422A Sgk



HA-T256A/S422A  
Sgk



**A**



**B**

